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STUDIES ON *CORYNEBACTERIUM RENALE*

IV. Renal Lesions in Mice Produced by Other Bacteria

By

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INTRODUCTION

The intravenous injection of mice with large numbers of living *Corynebacterium renale* produces necrosis and inflammation of the medulla of the kidney with no primary involvement of the renal cortex (Lovell and Cotchin, 1946). To determine whether the lesion produced can be regarded as specific, a preliminary study of the occurrence and distribution of lesions in the kidneys of mice after the intravenous injection of a number of other organisms has been made.

METHODS

The following bacteria were used in the investigation.

Corynebacterium ovis. A toxinogenic organism causing caseous lymphadenitis in sheep and ulcerative lymphangitis in horses.

Corynebacterium pyogenes. A toxinogenic organism associated with suppuration in a number of animal species; it may be recovered occasionally from pyæmic nephritis in cattle.

Bacterium equirulis. A common cause of pyæmic nephritis in foals.

Bacterium coli (human origin).

Staphylococcus aureus (toxinogenic and haemolytic).

Different strains of mice were used. They were 4 to 6 weeks old, and, as far as possible in equal numbers of males and females. Because of their age it is unlikely that any of the mice were affected by the renal lesion described by Dunn (1949) which simulates chronic pyelonephritis in man and is observed in some old mice accompanied by amyloidosis.

All inocula were saline suspensions of living bacteria.

OBSERVATIONS AND RESULTS

Corynebacterium ovis. Sections of kidneys were examined from 14 mice which had been inoculated intravenously with *C. ovis.* The dose varied from 1000 million to 2000 million living organisms grown on blood agar plates incubated at 37°C for 24 to 48 hours. Eight of the mice died during the first four days, three were killed on the fifth day and three on the tenth. Renal lesions were present in all. The cortex was involved in each case; the medulla was diseased in ten of the mice but more may have been affected as only a few sections of each kidney were cut. In general the region most markedly affected was the upper portion of the medulla. The mice which died during the early period

showed in sections large clumps of bacteria in the kidney blood vessels with little or no sign of local necrosis or inflammatory reaction. These clumps were seen mainly above the prominently vascular zone in the medulla and in the glomerular capillaries of the cortex. (Figure 1). From the third day onwards local necrosis was observed, abscesses were seen in the cortex and in the medulla and pus was present in the renal pelvis.

Corynebacterium pyogenes. The number of mice whose kidneys were examined in this group was 27. Six received a suspension prepared from serum broth cultures, nine were inoculated with a similar suspension from the growth on blood agar plates, and 12 received washed suspensions from 24 hour-cooked-meat broth cultures care being taken to eliminate any trace of *C. pyogenes* toxin. The doses given varied from 2,000 million to 7000 million organisms. Eight mice died between the first and tenth day and the remainder were killed at intervals up to the 21st. day. Ten of the 27 mice showed renal lesions; cortical lesions were present in six of them and medullary lesions in at least eight. The lesions comprised focal accumulations of inflammatory cells with suppuration; pus was observed in the renal pelvis and the upper end of the ureter was inflamed. There was a large necrotic core in the medulla in one mouse (Fig 2).

Bacterium equirulis. Six mice were given approximately 2000 million living *Bact. equirulis* and all were dead within two days. Five showed renal damage and clumps of organisms were found in the cortical vessels and glomeruli; masses of bacteria were also observed in vessels just below the more vascular zone of the medulla. A further ten mice were inoculated with smaller doses, some with about 900 million and some with 400 million organisms. Renal lesions were not found in one mouse that died within one day of inoculation nor in the survivors which were killed after five, seven, twelve and 105 days.

Bacterium coli. The 22 mice from which kidney sections were examined had received *Bact. coli* prepared from cultures on agar; the doses varied from 750 million to 4000 million. Eight of the mice had died by the fourth day and the others were killed at intervals up to the 21st. day. Renal lesions were found in three of the mice which died and in five of those which were killed; seven showed cortical damage and four had changes in the medulla. In one mouse there were medullary changes with no observable lesions in the cortex. In general, the cortex was more prone to damage and the cortical blood vessels and glomeruli examined within a day or so of inoculation contained clumps of bacteria: similar masses were seen occasionally in the medulla. Inflammatory and suppurative lesions developed in the cortex (Fig. 3), and at a later stage in the medulla. In one case the medullary lesion resembled that induced by *C. renale.* Thrombosis of branches of the renal veins also occurred.

Staphylococcus aureus. The kidneys of 57 mice were examined. The dose injected varied from 100 million to 2000 million living organisms. Fourteen out of 17 mice which died and 29 out of 40 which were killed between the second day and the end of the third month after inoculation, a total of 43, had renal lesions. Lesions were found in the cortex in 36 and in the medulla in 38. The cortex alone was affected in five, the medulla alone in seven and both cortex and medulla in the remainder. The changes observed consisted of necrotic and suppurative foci (Fig. 4). In 21 of those examined the apex of the medulla showed a necrotic lesion similar to that found in the experimental infection of mice with *C. renale* (Fig. 5). There was a cystitis in one mouse (Fig. 6). Most of the mice were dead or had been killed by the seventh day but there were a few survivors and these, when killed two to three months later, showed suppurative foci in many sites other than the kidneys such as the liver, lungs, pericardium, myocardium, subarachnoid space of the spine, connective tissues and muscles around some of the joints and the bone marrow. In two mice examined on the 69th. and 88th. day respectively lesions were found in other organs in the absence of any observable kidney damage.

TABLE I.

NUMBER OF MICE IN WHICH RENAL LESIONS WERE OBSERVED AFTER THE INTRAVENOUS INOCULATION OF LIVING SUSPENSIONS OF DIFFERENT BACTERIA.

	Numbers of mice				
	Injected	*Died*		*Killed*	
		+	−	+	−
C. ovis	14	8	0	6	0
C. pyogenes	27	4	5	6	12
Bact. equirulis	16	4	1	1	10
Bact. coli	22	3	7	5	7
Staph. aureus	57	14	3	29	11

+ = lesions in kidneys.
− = no lesions in kidneys.

DISCUSSION

It is clear that the intravenous administration of several different species of bacteria may in a proportion of mice induce lesions in the kidneys (Table I). None of the organisms, however, produced lesions which were confined exclusively to the medulla. It is true that *Staph. aureus* induced medullary changes which were similar to those seen with *C. renale* (Lovell and Cotchin, 1946) but some of the mice in this series showed, in addition, cortical changes. In so far as these studies have progressed it appears justifiable to regard as specific the localization of *C. renale* in the renal

medulla of the mouse after intravenous inoculation; it is of course possible that further comparative studies will reveal other bacteria capable of inducing this reaction. The purpose of the work recorded here was to elucidate this point and the species of bacteria administered were selected with this object in view. It has been demonstrated that these bacteria can and do produce lesions in mice after intravenous inoculation. *C. ovis* caused kidney changes in all the mice inoculated with this organism, *Staph. aureus* in over two-thirds, and *C. pyogenes, Bact. equirulis* and *Bact. coli* in about one-third. In general, the early changes consisted of the accumulation of bacteria in the capillaries of the cortex and medulla; there was at this stage no definite local reaction though necrosis and suppuration appeared later. The appearances at times suggested that with these infections the medullary lesions were produced first and that some of the cortical lesions had spread from them.

SUMMARY

The intravenous injection of mice with living suspensions of *C. ovis, C. pyogenes, Bact. equirulis, Bact. coli* and *Staph. aureus* produced renal lesions in a variable proportion of animals. These lesions differed in distribution from those which were observed after the similar administration of living suspensions of *C. renale*, as they were not confined to the medulla but were found also in the cortex. It is therefore concluded that, as far as our observations extend, the distribution of the lesions produced by *C. renale* are specific for that bacterium.

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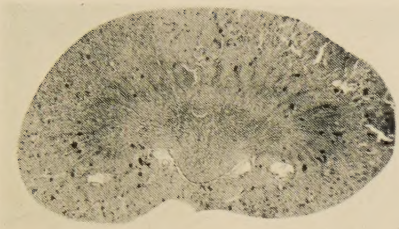

FIG. 1.

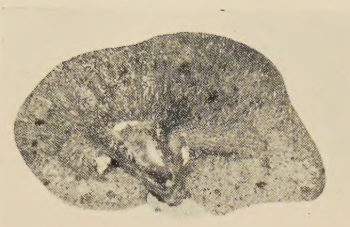

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

FIG. 1. Kidney of mouse with clumps of organisms scattered throughout cortex and upper part of medulla 5 days after inoculation with *C. ovis*. Gram. ×6.

FIG. 2. Kidneys of mouse with pyelonephritis, necrosis in medulla and minor lesions in cortex 11 days after inoculation with *C. pyogenes*. Haematoxylin and eosin. ×5.

FIG. 3. Kidney of mouse with cortical abscesses and thrombophlebitis in the branches of the renal vein 4 days after inoculation with *Bact. coli*. Haematoxylin and eosin. ×6.

FIG. 4. Kidney of mouse with suppurative lesions in the medulla and radial streaks of suppuration in the cortex 7 days after inoculation with *Staph. aureus*. Haematoxylin and eosin. ×5.

FIG. 5. Kidney of mouse with abscesses in medulla and slight lesions only in the cortex; marked necrosis of apex of papilla with large clumps of organisms 7 days after inoculation with *Staph. aureus*. Haematoxylin and eosin. ×6.

FIG. 6. Bladder of mouse with chronic suppurative inflammation 19 days after inoculation with *Staph. aureus*. Haematoxylin and eosin. ×6.

VACUOLATED CELLS IN THE DORSAL ROOT GANGLION OF THE RABBIT

By

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INTRODUCTION

During an investigation into the invasion of the nervous system by certain neurotropic viruses, serial sections of a large number of dorsal root ganglia of infected rabbits were prepared. About two thirds of the ganglia, whether they showed lesions or not, were found to contain one or more vacuolated cells. Similar cells were present in Gasserian ganglia. On examination, normal control rabbits were also found to have such cells in their spinal and Gasserian ganglia. Because of the resemblance of these cells to the ballooned cells met with in the nervous system of scrapie sheep it was thought worth while drawing attention to their normal occurrence in the rabbit, especially as none of the larger works on neurohistology remarks upon them.

MATERIAL AND METHODS

Altogether 197 spinal ganglia and 78 Gasserian ganglia from sixty-five animals infected with rabies, herpes or pseudorabies (Aujeszky's disease), have been sectioned. In addition twenty normal rabbits have been examined. As a rule one ganglion from each spinal region was examined. Some were fixed in Zenker's fixative without the addition of either formol or acetic acid: others were fixed in Bouin's mixture or in formol. Sections were stained with Giemsa or hæmatoxylin and eosin. Fat stains and the Bauer test for glycogen were also employed. Ganglia have also been examined from man (2), macaque (4), sheep (3), pig (1), lynx (2), dog (2), cat (2), rat (3) and chicken (1), but in no case have vacuolated cells been observed.

FINDINGS

Vacuolated cells of the type shown in figure 1, were met with in 143 out of the 197 ganglia examined from virus infected animals (72·6 per cent): 14 of the twenty normal rabbits showed similar cells (70 per cent.). They were more numerous in the lumbar and cervical than in the thoracic regions, perhaps because of the larger number of cells in these ganglia. No attempt was made to estimate the actual proportion of vacuolated cells in any given ganglion. No sex difference was noted nor did these cells appear more numerous in older animals. They were found in rabbits bought at random from dealers in this country and at the Institut Pasteur in Paris and also in laboratory bred animals. The vacuole was

usually a large single structure but sometimes was subdivided into three, four or more loculi. Usually it appeared empty but occasionally contained a little amorphous, finely granular, eosinophilic material. No fat or glycogen was found in the vacuoles. These cells occurred in all parts of the ganglion, immediately beneath the capsule as well as in its interior and were characteristically found lying side by side with cells in perfect preservation (Fig. 1). The nucleus and Nissl substance within vacuolated cells were always normal. It was unusual to find more than one or two such cells in a single section of a whole ganglion and unless serial examination were carried out they might readily be mistaken for shrinkage spaces.

DISCUSSION

None of the larger reference books of neurohistology or neuropathology make reference to these cells as normally occurring. Their existence has been noted by van Gehuchten and Nellis (1898) who observed them consistently in the spinal ganglion of the rabbit but do not record the number of observations made. Cells with 10 or 12 vacuoles were seen, the cytoplasm being reduced to fine partitions between adjacent vacuoles. Because van Gehuchten and Nellis found these cells after different methods of fixation they discarded the possibility of their being artefacts. The excellent preservation of adjacent cells in the present material supports their belief. The authors remark that what appeared strange to them was that " ces cellules n'ont pas encore été décrites " (p. 377). Even to-day the occurrence of these cells is not acknowledged in exhaustive descriptions (e.g. von Moellendorff 1928). Apparently similar cells are occasionally encountered in the spinal ganglion of the normal sheep though they are more frequent in animals affected with scrapie (Brownlee, 1940). Motor nerve cells showing the same vacuolated appearance whilst preserving their Nissl substance intact are met with characteristically in the medulla of scrapie sheep (Fig. 2). They are mentioned by Holman and Pattison (1943) and are also seen on rare occasions in sheep dying from obscure wasting conditions (" pine "). Their significance is not known though somewhat similar cells are figured by Buzzard and Greenfield (1921) as occurring in chronic alcoholism in man. Andrew (1941) produced some degree of vacuolation of brain cells in the mouse by starvation but such cells are different in appearance from those here described. It is possible that vacuole formation is associated with some degree of irritation of the cells, for considerable numbers are to be found in the sciatic ganglia after inoculation of herpes virus directly into the nerve. Similar vacuolation also occurs sometimes in the opposite ganglion (Fig. 3). Even though there is considerable small round cell infiltration and capsular cell proliferation many of the vacuolated cells have preserved their Nissl substance remarkably well.

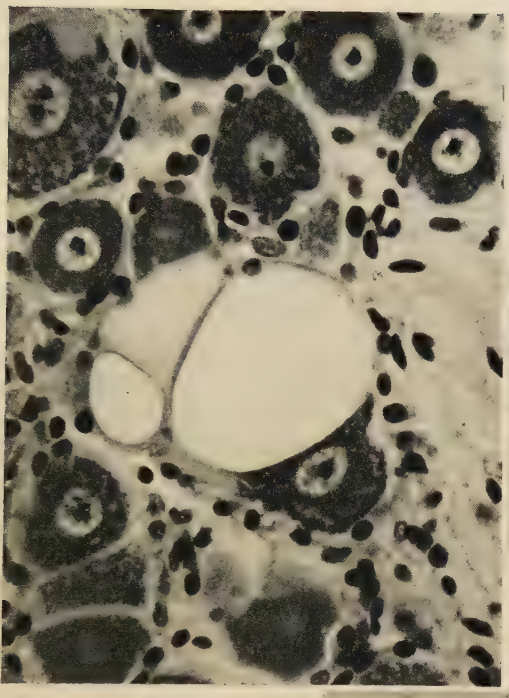
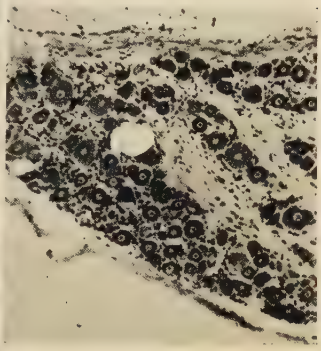
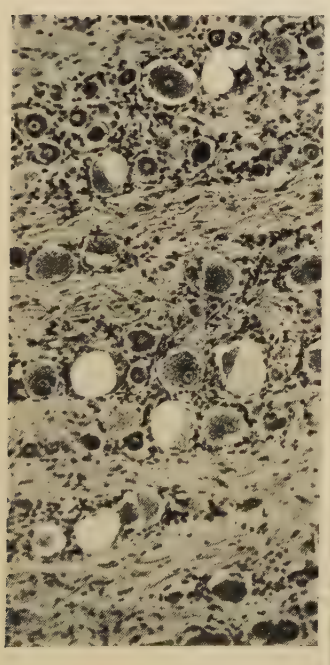
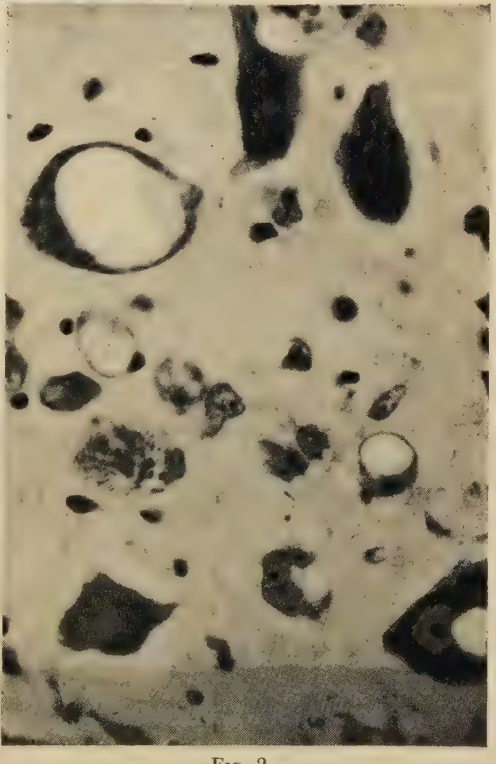


FIG. 1 (b)

FIG. 1 (a)







[FIG. 3

FIG. 2

FIG. 1 (*a*) Lumbar dorsal root ganglion of a normal adult male rabbit, Zenker fixation. Giemsa stain. Two vacuolated nerve cells are visible. × 65.
(*b*) The central vacuolated cell of Fig. 1(*a*). Note normal nucleus and tigroid substance. × 470.

FIG. 2. Medulla of scrapie sheep. Medial vestibular nucleus. Formalin fixed. Haematoxylin and eosin. Vacuolation of same nerve cells with preservation of normal nucleus and tigroid substance. × 550.

FIG. 3. Right lumbar dorsal root ganglion of rabbit inoculated with herpes virus into left sciatic nerve (E. 325). There is moderate small round cell infiltration and eight vacuolated cells are visible. Nissl substance is preserved in these cells. × 95.

True fatty degeneration has been found in the sensory neurones of man and other animals in old age (Truex and Zwemer, 1942), but the vacuoles described in the present material presented no fatty contents when examined in frozen sections specifically stained, and are presumably of a different nature.

Whilst no certain explanation of vacuole formation can be offered it seems possible that in the rabbit it is a response to excessive stimulation and may perhaps represent a phase in normal activity. It is possible that discharge of vacuole contents may lead to the formation of the small, rather deeply staining cells which are prominent in the rabbit spinal ganglion.

SUMMARY

Vacuolated nerve cells are a feature of the spinal ganglion of the normal rabbit in about two thirds of animals. They may result from unusual activity of the cells. Their similarity to vacuolated nerve cells met with in scrapie of sheep is indicated.

ACKNOWLEDGMENTS

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FERMENTATIVE ACTIVITIES OF SOME MEMBERS OF THE NORMAL COCCAL FLORA OF THE HORSE'S LARGE INTESTINE

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INTRODUCTION

The high incidence of diseases of digestion in the horse has long been recognised. Dobberstein and Dinter (1941) found that of 1121 autopsies on horses they accounted for 49·4 per cent. In view of this fact it is surprising that fundamental information on the physiology of digestion in the horse is conspicuously lacking. One reason for this has been the surgical problems involved in preparing fistulæ into the various parts of the bowel. However, some of these problems have already been surmounted. Alexander and Donald (1949) described a technique for the fistulation of the cæcum and large colon, and more recently Alexander (1952) has found it possible to prepare a horse with fistulæ in the cæcum and dorsal and ventral colons. By means of such fistulated animals observations on the fermentative processes of the large bowel have been made, and some of the more active bacteria involved have been isolated.

Particular attention has been paid to cocci in view of the predominantly coccal microflora of other alimentary sites, such as the rumen of sheep and cattle (Hungate, 1950; Sijpesteijn, 1951), where active carbohydrate fermentation is proceeding. It was hoped also to throw some light on the anomalous behaviour of *Streptococcus equinus* (Andrewes and Horder, 1906) the characteristic streptococcus of horsedung, which ferments but few sugars when compared with other fæcal streptococci (Orla-Jensen, 1943). By standard enrichment procedures several facultatively anærobic streptococcal strains of wide fermentative powers have been isolated from colon sites in fistulated horses and also a Gram-negative coccus, which is very active in fermenting lactate, a normal fermentation product of fæcal streptococci. This paper deals in detail with the fermentative behaviour of these bacteria and with the question of their identification.

METHODS

Experimental animals. Three ponies were used, two (B and G) having fistulæ in the right dorsal and ventral colons (hereinafter referred to as colons 4 and 1 respectively) and the third (T) fistulated in colon 1 only. The animals were maintained on hay and oats throughout.

Enrichment of bacteria in the colon contents. Most attention was paid to the fermentation of lactose, and of cellobiose a possible

intermediate in cellulose degradation, which are not usually attacked by *Str. equinus*. Material from the colon was withdrawn in the morning, collected in warm and chemically clean beakers, taken immediately to the 39° incubator and strained through 4 layers of clean, sterile surgical gauze in order to remove the greater part of the plant debris. The filtrate was quickly distributed in 20 ml. lots, into sterile 6″ test-tubes, each containing not more than 200 mg. of the solid, finely powdered sugar to be fermented previously sterilised by steam. After thorough mixing to dissolve the sugar, each test-tube was fitted with a clean and steam-sterilised cork bearing a bunsen-valve attachment, and all, save the unfermented controls which were immediately put in the cold room, were then incubated for 6 to 7 hours, when the fermentation was stopped by cooling to 0°. It was considered advisable not to continue the fermentations for a longer period in order to avoid an appreciable growth of chance contaminants.

Analysis of fermentation liquors. The pH as determined by the glass electrode was a sufficient indication of a good fermentation. It was invariably found that at values below pH 5.0 most of the sugar had disappeared. Lactic acid was determined colorimetrically (Hillig, 1942) and the total volatile fatty acid by repeated steam distillation after acidification. In some instances a viable bacterial count was made, both before and after fermentation in 1 per cent Bacto Yeast Extract agar medium containing 1 per cent lactose or cellobiose, by the roll-tube method. The roll-tubes were incubated for 2 days at 39° in a N_2+5 per cent CO_2 atmosphere. Colonies which were almost invariably cocci were then picked off for microscopic examination and purification by replating in the usual way.

Isolation of lactate-fermenting bacteria. A highly-diluted colon liquor was judged to contain lactate-decomposing bacteria if marked disruption occurred in shake agar tubes after 24 hours incubation. The medium was composed of Na lactate, 2 per cent; Bacto Yeast Extract, 0·67 per cent; Bactopeptone, 0·33 per cent; K_2HPO_4, 0·33 per cent; KH_2PO_4 0·1 per cent; agar 2 per cent and sodium lactate as the source of carbohydrate. The initial pH was between 6 and 7. A control with no lactate in the medium never showed gas production even after several days' incubation.

For the isolation of the lactate fermenting bacteria, sterile Pasteur pipettes were inserted into the bottom of the disrupted shake cultures, where there was usually a free space containing some turbid liquid, and a few drops withdrawn for microscopic examination. If found to be rich in Gram-negative cocci, the rest of the liquid was transferred to a second shake culture from which blood agar plates incubated anærobically at 40° were prepared. Purification was carried out by alternate culture of isolated colonies in liquid lactate medium, followed by replating on blood agar. When only Gram-negative diplococci were seen in stained

films and the final isolate also disrupted lactate agar, it was concluded that a pure culture had been obtained.

Serological methods used for identification. Precipitin tests were carried out with extracts made both by the acid method of Lancefield (1933) and by formamide extraction as described by Fuller (1938). The following group specific antisera were available: A, B, C, D, E, F, G, H, and K from Burroughs Wellcome & Co., and N from Dr. Mattick, Shinfield, Reading. Most useful information was obtained by the application of the Neufeld 'capsular-swelling' reaction used by Mirick, Thomas, Curnen and Horsfall (1944) for *Str. salivarius* and related strains.

Antisera homologous to the newly isolated horse streptococci were prepared by the method of Lancefield (1934). Cultures for injection into rabbits were prepared by re-suspending cells from 18 hour broth cultures in 0·85 per cent NaCl, containing 0·2 per cent formalin, to a volume equal to 5 per cent of that of the original culture. After 48 hours at 0° the bacterial suspensions were sterile. Immediately before use suspensions were diluted 20 times with saline. Doses of 1 ml. were injected intravenously into rabbits daily for 1 week, followed by a week's rest. Three courses of injections were given.

RESULTS

Properties of Colon 1 and Colon 4 Liquors

The respective micro-fauna of these liquors are quite distinct and serve to differentiate between them (Strelkow, 1939; Adam, 1951). There are also differences in chemical and physical properties. Strained colon 4 liquor is in general thicker and darker in colour than colon 1 liquor, and contains rather more material precipitable by dilute acetic acid (mucoprotein?). Both are essentially phosphate buffers (equivalent to about 0.15 per cent K_2HPO_4) and are neutral in reaction. Colon 1 liquor also appears to contain a little bicarbonate, since its pH rises to 8·0-8·5 on standing. In each instance the buffering capacity is heightened by the pressure of appreciable concentrations of volatile fatty acid anions.

Sugars other than lactose fermented by colon contents

Pentoses were not readily attacked. Cellobiose, fructose, glucose, maltose, starch and sucrose were utilised by both colon 1 and colon 4 liquors, e.g. 0·25 per cent of dissolved substrate disappeared in 6 hours. Galactose and lactose were more readily attacked by colon 1 than by colon 4 liquor.

Lactose fermentations by colon 1 and colon 4 liquors

In these experiments the colon 1 contents were from Pony T and the colon 4 contents from Pony B. The results of two separate 7 hour fermentation tests are summarised in Table I. Isolations of cultures were made from the colonies developing in the viable

counts underlined in Table I with results summarised in Table II. The seven streptococcal isolates denoted by asterisks in that table were kept in cultivation and a more extended study made of their fermentation reactions. Three of these strains were kept for the serological study recorded later.

TABLE I

RESULTS OF FERMENTATION OF LACTOSE AND OTHER CARBOHYDRATES BY COLON LIQUORS 1 AND 4.

Experiment	*Carbohydrate*	*Final pH*		*Viable bacterial count on lactose-yeast extract-agar (per ml ; × 10⁶)*	
		Colon 1	*Colon 4*	*Colon 1*	*Colon 4*
I	None (after incubation)	7·0	No test	No test	1
	Lactose	4·85	5·05	*68·75*	*420*
	Cellobiose	4·7	4·6	150*	87·5*
	Mannitol	6·7	6·7	No test	No test
II	None (before incubation)	6·6	7·2	*2*	*2·5*
	None (after incubation) ...	7·0	6·8	No test	No test
	Lactose	4·7 ; 4·7	5·7 ; 5·8	*90*	*15*
	Cellobiose	4·6 ; 4·5	4·9 ; 4·8	No test	164·5*

*On cellobiose-yeast-extract agar.

TABLE II

NUMBER OF LACTOSE AND NON-LACTOSE FERMENTING STREPTOCOCCI FROM ISOLATED COLONIES.

Source of streptococci and kind of fermentation liquid examined	*No. of colonies picked off*	*No. of lactose-fermenting strains isolated*
Colon 1 ; lactose	5	5
Colon 4 ; lactose	9	7
Colon 1 ; before fermentation	7	6*
Colon 1 ; lactose	1	1*
Colon 4 ; before fermentation	2	0
Colon 4 ; lactose	3	0

* Kept in subculture.

Properties of the lactose-fermenting streptococci. These streptococci were short-chained or diplococcal in appearance, the chains being embedded in a thin mucoid material, particularly on solid media. The strains were non-haemolytic and only one strain which quickly died out in sub-culture, fermented mannitol. The others fermented arabinose, glucose, galactose, maltose, lactose, trehalose,

raffinose, starch, dextrin and æsculin with production of acid, chiefly *d-lactic*. After 5 days' incubation the pH which was initially 7·6 had fallen to between 4 and 5. The following were not fermented: xylose, rhamnose, glycerol, mannitol, dulcitol, sorbitol, inositol. One strain (J) fermented sucrose, one strain (A) fermented both inulin and salicin. The two strains (A, J) and a third (N) which gave typical fermentation reactions, were positive to the methyl red test, did not liquefy gelatin, hydrolysed Na hippurate and reduced methylene blue with clot in skim milk medium.

Antigenic relationships of the lactose-fermenting streptococci. Antigenic extracts prepared from strains A, J and N by the formamide method of Fuller (1938) gave positive precipitin reactions with homologous antiserum prepared against strain J. On the other hand they gave entirely negative results when tested against all commercial sera, or against antisera from sheep's rumen streptococci (MacPherson, 1952). These results were most convincingly confirmed by means of the Neufeld capsular swelling reaction. Only in the presence of the homologous antiserum was the capsule clearly outlined as a greyish zone surrounding each pair or short chain of cocci. The addition of antiserum J to the colon liquor itself revealed definite capsulation in a small proportion of the cocci present. With normal serum no such capsulation could be demonstrated (Figs. 1 and 2).

FIG. 1

FIG. 1. Horse colon streptococcus strain J and normal rabbit serum. No obvious capsule. × 2500

Lactate fermentations by colon 1 and colon 4 liquors

On all occasions with all 3 ponies disruption of the agar occurred when the colon 1 contents were diluted 600 times with

FIG. 2

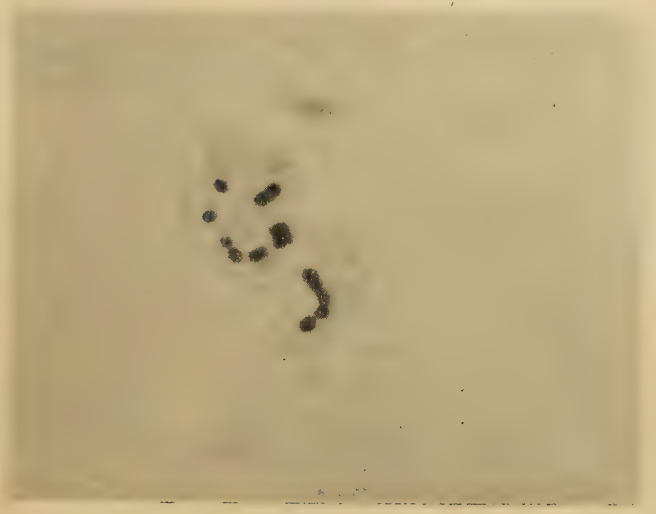

FIG. 2. Streptococcus J and homologous antiserum, showing clear delineation of the capsule. × 2500

Both were wet preparations stained with methylene blue.

sterile water before testing. On some occasions dilutions of 10,000 or even 25,000 gave a positive result. It was not always possible to demonstrate lactate-fermenting bacteria in colon 4 contents if the material was highly diluted but a positive result was always obtained if undiluted colon contents was used. If sterile Na or NH_4 lactate (0·35 per cent) was dissolved in the strained colon liquor and the fermentation carried on without agar for 7 hours at 39° there was evolution of gas with no appreciable change in pH. Volatile acid was produced and lactic acid disappeared as indicated in Table III. At the end of the fermentation period lactate-fermenting bacteria could be demonstrated at dilutions of 100,000 by the agar-disruption technique.

TABLE III

DECOMPOSITION OF LACTATE BY COLON 1 CONTENTS OF PONY B

Substrate	*Final pH after 7 hours' fermentation*	*Increase in volatile acid, calc. as acetic mg%*	*Loss of lactic acid mg%*
None	6·1	36	None
Na lactate	6·8	216	150
NH_4 lactate	6·2	198	220
Free lactic acid	6·2	114	150

Properties of the lactate-fermenting Gram-negative cocci. These organisms were strict anærobes with rather exacting growth requirements, blood agar or cooked meat medium being necessary for their continued propagation. No carbohydrates were fermented and among all the hydroxy and dicarboxylic acids tried as substrates, gas was produced only from lactate, and to a lesser extent from tartrate. The colonial appearance on agar and most other properties were exactly as described in detail by Johns (1951) for the organism obtained from the sheep's rumen, and presumed to be *Veillonella gazogenes*. In view of this and of the frequent occurrence of this coccus in intestinal sites no further investigation of our equine strain was considered necessary.

DISCUSSION

It is clear that the *Str. equinus* of fæcal origin, which ferments only a few carbohydrates, is by no means the only streptococcus to be found in the colon of the horse. The streptococci which we have isolated, in so far as fermentation reactions are concerned, resemble the well-known *Str. bovis* which has wide fermentative powers (Orla-Jensen, 1943), but on serological grounds seem to fall into a group of their own. They are capsulated and their presence, either in pure or in mixed cultures or in the colon fluids, can conveniently be demonstrated by means of the Neufeld capsular swelling reaction provided homologous antiserum is used. We are of the opinion that this very simple serological test might be more widely used in the identification of encapsulated streptococci in the natural situations in which they occur. A further study (MacPherson, 1952) along these lines is in progress with other animals.

The chief fermentation product of these colon streptococci is lactic acid which is not usually found in colon liquor. Consequently, the presence in the colon of the ubiquitous *Veillonella gazogenes*, which ferments no sugars but only lactate to volatile fatty acids is not unexpected. Since a more vigorous fermentative activity is observed in the ventral than in the dorsal part of the colon, the greater concentration of the Gram-negative coccus in the former site and the presence of bicarbonate ions in quantity in colon 1 liquor only is readily explained. It seems unlikely, however, that *V. gazogenes* can be responsible for the production of the greater part of the high volatile fatty acid content which has been estimated at 0·3 to 0·7 per cent of colon liquor (Alexander, 1952), partly because it seems to be present in insufficient numbers and partly because the colon does not normally contain large volumes of gas. The fermentation of lactate by *V. gazogenes* would undoubtedly produce hydrogen as well as CO_2 (Johns, 1951). It may be that lactate is very rapidly absorbed from the colon and only small amounts remain for fermentation with *V. gazogenes*. The fermentative and absorptive processes of the horse's colon evidently

need a much fuller study, which one of us (F.A.) hopes to carry out in the near future.

A point of some interest is the fact that the common streptococcus of horse dung differs so markedly from the organisms isolated from the colon. It is possible that selective feeding on the part of ciliate protozoa, which are present in large numbers in colon 4 (Adam, 1951), may account for this anomaly.

SUMMARY

Streptococci capable of fermenting lactose and starch have been isolated from the large colon contents of fistulated ponies.

They form a capsule which is easily demonstrated by the capsular swelling reaction described by Neufeld.

They can be differentiated serologically from other types of streptococci.

The colon also contains the Gram-negative coccus *Veillonella gazogenes*, an anærobic fermenter of lactate.

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THE CHARACTERS OF STREPTOCOCCI ISOLATED FROM THE UTERI OF THOROUGHBRED MARES IN IRELAND

By

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INTRODUCTION

The occurrence of streptococcal uterine infections in thoroughbred mares in Ireland has been known for a number of years and has been referred to by Craig (1931) and by Kearney (1948, 1950), but no detailed description of the streptococci concerned has been recorded. An early observation on the occurrence of streptococci in the genital tracts of mares was made by Ostertag (1901) who found the organisms to be the cause of abortions at several German studs. He recovered streptococci both from aborted foetuses and from the surface of the allantois-chorion. Zwick (1913) described the finding by investigators at the German Imperial Office of Health of streptococci, among other organisms, in the bodies of aborted foetuses from a German stud. Dimock and Edwards (1928), in a bacteriological survey of 1,606 mares in the United States, recovered streptococci from the genital tracts of 24 per cent of the mares examined. Bazely and Battle (1940) studied the characters of 457 strains of streptococci isolated from horses. All were beta haemolytic and all gave positive precipitin reactions with Lancefield's Group C anti-serum when tested by the micro-acid-extract method. All strains isolated from the genital tracts of mares fermented lactose and sorbitol, but not trehalose.

As has been indicated, streptococci have been isolated frequently from the genital tracts of mares and the characters of the organisms have received some attention from a number of investigators. Lack of this information in respect of thoroughbred mares in Ireland suggested the need for the present investigation.

MATERIAL AND METHODS

Strains of streptococci were isolated from 48 uterine swabs taken from thoroughbred mares. The technique employed in obtaining the swabs was as follows: — The mare was held facing inwards in a loose box with her hindquarters in the doorway. The tail was bandaged and the perineal region was washed with warm water. No soap or disinfectant was used. A sterile vaginal speculum was inserted and opened. By means of 16″ forceps a sterile swab was introduced into the os uteri, care being taken not to allow it to

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come in contact with any surface other than the cervical mucous membrane. The swab was rotated and moved in and out of the os uteri a few times before it was withdrawn and replaced in its tube. The test tube swab container was held mouth downwards by an assistant to prevent aerial contamination until the used swab was returned to it. On arrival at the laboratory, swabs were moistened in tubes of sterile glucose broth medium, stroked on two ox blood agar plates and returned to the tubes of broth. The tubes and plates were incubated at 37.5°C. in the dark for 48 hours. They were examined for evidence of growth at 24 and 48 hours. When satisfactory growth was obtained on a plate, a colony was picked off and transferred to glucose broth and incubated for 18 hours. From this culture subcultures were made into media containing various carbohydrates as enumerated below, into serum media for haemolysin tests and into flasks of glucose broth for the making of extracts for precipitin tests.

The following media were used in the investigation: —Glucose broth (0.5 per cent). Peptone water media containing 1.0 per cent Andrade's indicator and 1.0 per cent of one of the following carbohydrates: —lactose, salicin, sorbitol, and trehalose. Litmus milk. Serum media consisting of glucose broth containing 15.0 per cent ox or horse serum. Blood agar medium containing 5.0 per cent citrated ox blood.

RESULTS

A total of 48 strains of streptococci were recovered from the uteri of thoroughbred mares and examined with the following results.

Cultural results

Glucose broth medium. All strains grew readily in this medium and after 24 hours' incubation most strains produced a heavy deposited growth with clear supernatant fluid. A few strains produced uniform turbidity of the medium.

Blood agar plates. The colonies were small, shiny, hemispherical and surrounded by wide zones of haemolysis up to 4 mm. in diameter.

In most instances, streptococci were obtained in pure culture on plates and in tubes, but when swabs were contaminated, pure cultures were readily obtained on plating and subcultivation.

Biochemical results

In these experiments tests on the ability of strains to ferment carbohydrates were made by pipetting four drops of 18-hour glucose broth cultures into tubes of carbohydrate media and noting the results in these after five days' incubation. All strains fermented lactose, sorbitol and salicin. No strain fermented trehalose. All strains acidified litmus milk and produced a soft clot.

Serological results

Precipitin tests. These tests were carried out using the method of Lancefield (1933) slightly modified. The formolised culture was suspended in half the volume of saline recommended by Lancefield and a correspondingly smaller volume was used for intravenous injection of rabbits in the preparation of immune sera. Two courses of seven daily injections with an interval of ten days between the courses proved satisfactory for the production of antisera. The precipitin test itself was modified to a simple one tube method. Three drops of serum were placed at the bottom of a Dreyer's tube and an equal volume of extract was layered on the surface of the serum. Extracts from all strains were tested with sera produced against two of the strains and with a commercial Group C antiserum. A positive result was obtained in all the tests, a dense white ring appearing within one minute at the junction of bacterial extract and serum.

Haemolysin tests. Strains were tested for soluble haemolysin production by growing them in 15 per cent horse serum broth and in 15 per cent ox serum broth, using the method described by Minett and Stableforth (1931). Tests were done in duplicate using horse cells and ox cells for each strain tested. The lysin titres varied from 1 in 25 to 1 in 200 for complete haemolysis and from 1 in 50 to 1 in 1,600 for incomplete haemolysis. Generally, the higher titres were given by strains grown in ox serum medium tested against ox cells. The average titres for complete and incomplete haemolysis respectively in respect of ox serum broth and horse serum broth cultures tested with ox and horse cells were as follows: —

Ox serum broth cultures and ox cells	1/50 and 1/800
Ox serum broth cultures and horse cells	1/50 and 1/400
Horse serum broth cultures and ox cells	1/25 and 1/200
Horse serum broth cultures and horse cells	1/25 and 1/200

The haemolysin titres recorded are lower in most cases than those obtained by Minett and Stableforth (1931) for complete haemolysis, i.e. up to 1 in 190, for strains grown in horse serum broth.

Pathogenicity tests

In these experiments the pathogenicity of strains was tested on rabbits and guinea pigs. The strains were grown in 15 per cent bovine serum glucose broth for 24 hours and doses of whole culture and washed culture deposit resuspended in fresh unincubated culture medium were given either intravenously or intraperitoneally to rabbits and intraperitoneally to guinea pigs. Inoculated animals which died were subjected to complete necropsy and bacteriological examination.

Pathogenicity for rabbits. The pathogenicity for rabbits of 7 strains was tested by the intravenous inoculation of one rabbit for each strain with 3 ml. whole culture. All the animals died within

5 days. Further tests were made in the case of five of these seven strains by the intravenous inoculation of one rabbit for each strain with the washed deposit of 3 ml. whole culture resuspended in fresh medium. All the animals died within 5 days. Of the 12 rabbits subjected to intravenous inoculation, 7 (58.4 per cent) died within 24 to 48 hours; 3 (25·0 per cent) died within 2 to 4 days and 2 (16.6 per cent) died 5 days after inoculation.

The remaining 41 strains were tested for their pathogenicity for rabbits by the intraperitoneal inoculation of one rabbit for each strain with 1 ml. whole culture. Of these 41 rabbits 19 (48.8 per cent) lived. Of the remaining 22 rabbits (51.2 per cent) which died, 16 (72.7 per cent) died within 24 to 48 hours; 2 (9.0 per cent) died within 2 to 4 days; 2 died within 4 to 7 days and 2 died 15 days after inoculation.

Animals which had been inoculated intravenously showed little gross change on post-mortem examination. Usually there was only slight splenic engorgement, some petechiae in the kidneys and pericardial effusion. Pulmonary congestion was apparent in some animals. Rabbits inoculated intraperitoneally showed acute peritonitis with purulent effusion containing fibrin strands.

Pathogenicity for guinea pigs. The pathogenicity for guinea pigs of all 48 strains was tested by the intraperitoneal inoculation of one guinea pig for each strain with 3 ml. whole culture. Further tests on 5 of the strains were made by intraperitoneal inoculation of one guinea pig for each strain with the washed deposit of 3 ml. whole culture resuspended in fresh medium. It was not possible to detect any difference between the whole culture and the washed deposit in respect of pathogenicity. Of the 53 guinea pigs inoculated, 23 (43.4 per cent) lived. Of the remaining 30 guinea pigs (56·6 per cent) which died, 15 (50 per cent) succumbed within 48 hours; 5 (16·6 per cent) within 2 to 3 days; 5 within 3 to 15 days and 5 within 15 to 21 days after inoculation. Lesions in the guinea

TABLE I

Animals	*Route of Inoculation*	*Material Inoculated*				*Mean time in days from inoculation to death*
		Whole Culture		*Washed Deposit*		
		Numbers		*Numbers*		
		Inoculated	*Died*	*Inoculated*	*Died*	
Rabbits	Intravenous	7	7	5	5	2·75
	Intra-peritoneal	41	22	—	—	3·0
Guinea-pigs	Intra-peritoneal	48	26	5	4	6·2

pigs consisted of a peritonitis varying in severity and type according to the survival time of the animal. Animals which died shortly after inoculation revealed an acute haemorrhagic peritonitis with effusion. Animals which survived for some days after inoculation showed adhesions and a purulent or sero-fibrinous exudate.

Bacteriological examination of all animals which died resulted in the recovery only of haemolytic streptococci in every case. The results of the pathogenicity tests are summarised in Table I).

DISCUSSION

The characters of the strains of streptococci isolated, as indicated by the results of the tests described, place them in Lancefield's Group C and Edwards's (1935) Type A (Type 2 of Bazely and Battle, 1940). This classification of streptococci recovered from the uteri of mares is in precise agreement with the findings of Bazely and Battle in Australia.

The results of the pathogenicity tests in rabbits appear to indicate some considerable variation in virulence from strain to strain and/or individual resistance to infection on the part of inoculated animals. However, pathogenicity of the strains for rabbits has been shown clearly and this confirms the findings of Dimock and Edwards (1928) on strains of streptococci isolated from mares in the United States of America. Minett and Stableforth (1931) found a similar variation in virulence in respect of *Streptococcus pyogenes*. Many of the rabbits used for intraperitoneal inoculations were very young and apart from possible variation in the virulence of infecting strains, it is possible, in view of the results obtained by de Gara (1945), that non-specific sensitisation to streptococci prior to the test inoculation rendered such animals less susceptible to experimental infection.

The results of intraperitoneal inoculation of guinea pigs differ remarkably from those obtained by Dimock and Edwards (1928) who found streptococci isolated from mares non-pathogenic for guinea pigs when administered by any route. Minett and Stableforth (1931) found *Streptococcus pyogenes* pathogenic for guinea pigs and described death from acute peritonitis.

While the pathogenicity for laboratory animals of strains of Lancefield's Group C streptococci isolated from the uteri of thoroughbred mares has been established, the precise role of these organisms in the pathogenesis of equine uterine infections remains obscure in view of the fact that they have been isolated from the uteri of apparently healthy mares during a period of nine days following normal parturition. In many of these animals the organisms disappeared after this time.

SUMMARY

The isolation of 48 strains of streptococci from the uteri of thoroughbred mares in Ireland has been described and the results of tests made on them recorded.

The pathogenicity of the strains isolated and the production of soluble haemolysin were found to vary considerably.

All strains were classified as being in Lancefield's Group C, Edwards's Type A (Bazely and Battle's Type 2).

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THE EFFECT OF PENOTRANE ON THE GROWTH *IN VITRO* OF *CANDIDA ALBICANS* AND *ASPERGILLUS FUMIGATUS*.

By

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INTRODUCTION

Moniliasis in turkeys and chickens occurs in the upper digestive tract and is associated with the presence of the fungus *Candida albicans* which is yeast-like and produces blastospores and pseudomycelia in tissues and exudates (Fig. 1).

Junghern (1933) described the condition in chicks and Hinshaw (1934) studied several outbreaks in chickens and turkeys in the United States. Buxton, Darcel, Gordon and Horton-Smith (1949) and Blaxland and Fincham (1950) have reported the condition in turkeys in Great Britain. Blaxland and Fincham considered that moniliasis occurs as a consequence of the presence of *C. albicans* in the crop in combination with environmental factors. Buxton *et al.* and Blaxland and Fincham have fully described the appear-

Fig. 1

Fig. 1. Blastospores and developing mycelium of *Candida albicans* obtained in washings of Sabouraud agar. ×1600.

ance at autopsy. The crop appears to be the principal focus of infection but the fungus has been isolated from other sites including the mouth, oesophagus, gizzard and intestines. In acute cases whitish ulcers of rosette form are distributed over the inner lining of the thickened wall of the crop.

Penotrane (Phenyl-mercuric-dinaphthylmethane-disulphonate) is superior to the quarternary ammonium compounds in that it is claimed to be equally active in the presence of serum and tissue debris. Goldberg, Schapero and Wilder (1950 a and b) showed that in broth containing serum concentrations of approximately 80 per cent. the activity of phenyl-mercuric-dinaphthylmethane-disulphonate is three times as great as that of mercuric chloride, phenyl mercuric acetate and silver nitrate respectively per unit of active cation content.

The compound was tested for activity against three Gram-positive and three Gram-negative organisms and was found to possess high activity against *Staphylococcus aureus; Streptococcus pyogenes, Bacillus subtilis, Escherichia coli, Pseudomonas pyocyanea* and *Proteus vulgaris.*

Goldberg *et al.* (1950a) studied the action of the compound in relation to its effect on *Aspergillus fumigatus* and *A. niger* as well as *Monilia (Candida) albicans.* These authors showed that when applied as an aqueous solution it penetrates the living epidermis, dermis and connective adipose tissue and enters into the subjacent muscle. No significant biochemical changes were noted in body tissues as shown by macroscopical and microscopical appearances. They also found that the compound is adsorbed upon tissue and builds up tissue concentrations greater than those in the solution originally applied.

The results obtained by Goldberg and his colleagues led to our testing the fungicidal properties of Penotrane against *C. albicans* and *A. fumigatus* of turkey and fowl origin when grown *in vitro.* We have not yet tested the compounds against natural outbreaks of moniliasis or in birds which have been experimentally infected. In one instance *Salmonella dublin* was used as a test organism.

METHODS AND RESULTS

The solid agar test

A 24-hour growth of the organism on Sabouraud's glucose agar in a petriplate was washed off with 10 ml. of saline. Sabouraud agar of 200 ml. volume was melted in a flask and allowed to cool to 50°C.: 10 ml. of suspension was then added and well mixed by careful rotation. The contents of the flask were poured into a trough of $5\frac{3}{4}''$ diameter $\times$ $2\frac{3}{4}''$ depth which provided a 15 mm. depth of solid agar. By the use of a sterile cork borer, holes of 7 mm. diameter were cut and the agar removed. The bases of the holes were sealed with two small drops of agar which was allowed to set. By this method 0.3 ml. Penotrane could be introduced into

FIG. 2

FIG. 2. Showing the inhibitory effect of various dilutions of Penotrane on (A) *Candida albicans* and (B) on *Aspergillus fumigatus*.

Dilutions of Penotrane used (1) 1/10,000, (2) 1/20,000, (3) 1/40,000, (4) 1/80,000, (5) 1/400,000, (6) 1/500,000. Note the occurrence of resistant colonies of *A. fumigatus* in (B).

each hole. The covered dish was then placed in an incubator at 37°C. for 24 hours before a reading of the radius of inhibition was taken.

The effect of the various dilutions of Penotrane used in this series on the growth of *A. fumigatus* and *C. albicans* is set out in Table I. This experiment took the form of a test in which the extent of the inhibitions of each organism was measured and compared. Some resistant colonies of *A. fumigatus* were observed within the areas of inhibition. These are clearly shown in Figure 2.

TABLE I

INHIBITION OF *Candida albicans* AND *Aspergillus fumigatus* IN VARIOUS DILUTIONS OF PENOTRANE.

Dilutions of Penotrane	*Radius of Inhibition mm.*	
	Aspergillus fumigatus	*Candida albicans*
1/ 5,000	14	10
1/ 10,000	13	8·5
1/ 20,000	10	8·0
1/ 40,000	8·5	7·5
1/ 80,000	6·5	7·0
1/ 160,000	4	3·5
1/ 320,000	Not readable as	2·5
1/ 640,000	resistant colonies	0·5
1/ 1,280,000	were present.	Nil
Control	Nil	Nil

Glucose-broth test

This method was much simpler than the plate test. Penotrane dilutions were made with glucose broth in tubes each containing 5 ml. of glucose broth. A loopful of 24-hour glucose broth cultures of *C. albicans* and *A. fumigatus*, or for *Salmonella dublin* which was used for comparative purposes, a loopful of a 24-hour peptone water culture was added to the Penotrane glucose broth tubes and incubated at 37°C. for 24 hours. A loopful from each tube was then subcultured on separate Sabouraud agar plates and incubated at 37°C. for 24 hours. The series gave an indication of the fungistatic concentrations.

This series of tests, which was comparative in nature, was designed to demonstrate the effect of Penotrane on the test organisms grown in glucose broth following subculture on to Sabouraud agar after incubation. The results are shown in Table II.

Penotrane thus proved to be bacteriostatic up to a dilution of 1/80,000 and fungistatic up to a dilution of 1/320,000.

Glucose broth plus horse serum

The tests with glucose broth incorporating serum were carried

TABLE II

VARIOUS DILUTIONS OF PENOTRANE IN GLUCOSE BROTH INOCULATED WITH TEST ORGANISMS.

Dilutions of Penotrane used	*Candida albicans*	*Aspergillus fumigatus*	*Salm. dublin*
	Subculture after 24 hrs.	*Subculture after 24 hrs.*	*Subculture after 24 hrs.*
1/20,000 : 80,000	—	—	—
1/160,000 : 320,000	—	—	+
1/640,000	+	+	+
Control	+	+	+

out as in the previous experiments, but serum at 10, 20 and 30 per cent. (v/v) was first added to the glucose broth before the dilutions were made with a view to studying the influence of serum on the fungistatic activity of Penotrane. Growth was observed after subculture on to Sabouraud agar. The results obtained in this series are shown in Table III.

TABLE III

EFFECT OF PENOTRANE ON *Candida albicans* IN SERUM GLUCOSE BROTH MEDIUM.

Dilutions of Penotrane	*Glucose Broth*			
	No serum	*Percentages of Horse serum added (v/v)*		
		10	*20*	*30*
	Growth after subculture			
1/ 2,500-80,000	—	—	—	—
1/160,000-320,000	—	+	+	+
1/640,000	+	+	+	+
Control	+	+	+	+

Contrary to other recorded findings there is some reduction in the activity of Penotrane against *C. albicans* in the presence of horse serum. At a dilution of 1/160,000 no apparent inhibitory action was observed in the presence of serum but in its absence a dilution of 1/320,000 was fungistatic.

Determination of the lengths of time required to inhibit the growth of C. albicans *with Penotrane at different dilutions*

A suspension of *C. albicans* from a 24-hour Sabouraud agar plate culture was made up in 20 ml. glucose broth. Dilutions of Penotrane were incorporated in this suspension in tubes, the times at which the drug was added were noted and the contents of the tubes were then well mixed by agitation of the tubes. As a preliminary, dilutions of Penotrane in glucose broth were left in the incubator at 37°C. and subcultures from the tubes were made every hour up to 11 hours after the Penotrane was added. As a check against possible contamination, a loopful of the culture, transferred to 10 ml. glucose broth, was incubated for 24 hours at 37°C. and thereafter subcultured on to solid medium.

In determining the lengths of time required to inhibit the growth of *C. albicans* with Penotrane at different dilutions, it was found that in the highest dilution used (1/100,000) there was complete inhibition after eight hours of exposure.

The results are shown in Table IV. These results show that with decreasing concentrations of the drug the period of exposure required to inhibit growth progressively increased.

Determination of the lethal effect of Penotrane on C. albicans

Penotrane was incorporated in glucose broth at dilutions ranging from 1/5,000 to 1/1,280,000. An overnight culture of *C. albicans* was washed off in 5 ml. saline and 0.02 ml. (approximately 10 million organisms as determined by plate count) was added to each dilution of Penotrane in glucose broth as well as to glucose broth without Penotrane. The tubes were incubated for 24 hours at 37°C. After this time, H_2S gas was passed through each Penotrane tube for two minutes and 0.02 ml. was then transferred to fresh glucose broth containing thioglycollic acid (0.1 per cent., v/v) previously neutralised to sodium thioglycollate. The cultures were then grown for an additional 24 hours at 37°C. The action of H_2S gas was to remove excess mercury from the medium, and sodium thioglycollate was added to the subculture media to remove any mercury which may have combined with the organic substances in the organisms. Subcultures from these tubes were grown on Sabouraud agar for 24 hours at 37°C. and examined.

After treatment for 24 hours with 1/80,000 Penotrane, *C. albicans* failed to grow when mercury-free inocula were subcultured on to Sabouraud agar. The fungus was recovered on subculture from dilutions of 1/160,000 Penotrane when freed from mercury and also from the untreated control tubes.

Determination of the effect of magnitude of inoculum on effective Penotrane concentrations

Dilutions of Penotrane ranging from 1/5,000 to 1/1,280,000 were incorporated in glucose broth as in previous tests. Tenfold

Table IV

Approximate Times in Which Dilutions of Penotrane Inhibited *C. albicans*.

Dilutions of Penotrane	*Recoveries of fungus after various times of exposure to different dilutions of Penotrane*																				
	Lengths of times of Exposure																				
	Minutes										*Hours*										
	½	1	2	3	4	5	10	15	20	30	1	2	3	4	5	6	7	8	9	10	11
1/ 10,000	+	+	−	−	−	−	−	−	−	−											
1/ 20,000	+	+	+	+	+	+	+	+	+	+	−	−	−		−	−	−	−	−	−	−
1/ 40,000											+	+	+	+	+	+	−	−	−	−	−
1/ 50,000											+	+	+	+	+	+	−	−	−	−	−
1/100,000											+	+	+	+	+	+	+	−	−	−	−

dilutions of a suspension of *C. albicans* in glucose broth from a 24-hour Sabouraud agar culture were made and 0·02 ml. of each dilution was inoculated into the series of tubes containing the dilutions of Penotrane. A Sabouraud agar plate was also inoculated with 0·02 ml. of the same suspensions and incubated for 24 hours at 37° C. The numbers of organisms contained in the inoculum were determined by plate count. A series of inocula varying from 125 to 12,500,000 organisms was thus made. After incubation 0·1 ml. from each test suspension was subcultured on to fresh glucose broth and grown for 24 hours and finally subcultured on to Sabouraud agar.

The magnitude of the inoculum had little effect on the fungistatic properties of Penotrane. Dilutions of Penotrane at levels of 1/5,000 to 1/320,00 in glucose broth successfully suppressed growth when inoculated with 125 to 12,500,000 *C. albicans* cells. Higher dilutions of Penotrane exhibited no fungistatic effect and the controls showed growth in all cases.

DISCUSSION

The fungistatic properties of Penotrane when tested *in vitro* warrant further investigation of this compound as a possible agent against epidemics of moniliasis in poultry in folds. Although *in vitro* tests of its effect on *Aspergillus* are satisfactory, the wide distribution of this fungus in the body of the chicken and its frequent occurrence in the respiratory organs, make it imperative that more must be known of the compound's behaviour and distribution in the body. Preliminary investigations suggest that its effect may be limited to the crop and intestinal tract of the chicken. In view of this observation, *in vivo* tests are now being undertaken to ascertain whether and to what extent the fungus in the crop wall might be affected by offering chickens dilutions of Penotrane in the drinking water. Chickens and turkeys will drink solutions of Penotrane at a concentration of 1/10,000 without apparent ill-effects.

SUMMARY

Tests carried out *in vitro* have shown that Penotrane (Phenylmercuric-dinaphthylmethane-disulphonate) is active against *Candida albicans* and *Aspergillus fumigatus* at a dilution of 1/320,000 and is probably fungicidal against the former species at a dilution of 1/80,000. There appears to be little correlation between size of inoculum and effective dilution of Penotrane.

Acknowledgment

We wish to thank Messrs. Ward, Blenkinsop & Co. Ltd. for supplies of Penotrane. We are also indebted to Dr. G. C. Ainsworth of the

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GASTRIC CARCINOMA IN THE DOG

By

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INTRODUCTION

Several writers (Wells, Slye and Holmes, 1938; Davis and Naylor, 1943; Willis, 1948; Nieberle and Cohrs, 1949) have emphasised how strikingly different is the incidence of gastric cancer in man compared with that in animals. In one series of 1,060 human cancers studied by Willis, 21 per cent originated in the stomach while several continental writers claim figures as high as 40 to 70 per cent.

Friedberger and Fröhner (1908), in 70,000 dogs examined post-mortem over a period of eight years, encountered only one gastric carcinoma which was not a primary tumour according to the statistical review of Sticker (1902). The latter author compiled a series of 766 cacinomas in the dog including a number traced from the literature as far back as 1860; the stomach was the origin in one case only, that of Eberlein (1897) which was a pyloric carcinoma with hepatic metastases. Magnusson (1915), Rubarth (1934), Wooldridge and Holmes (1935) and Davis and Naylor (1943) reported single cases of carcinoma of the pyloric region of the stomach. A review of tumours in animals was carried out by Dudzus (1940), and of 154 cases which he quoted, 14 occurred in the dog, but the number of carcinomas in this group is unknown to us, as the original compilation was destroyed during the recent war. Parascandolo (1901) carried out a resection for gastric cancer in a dog. Miles (1931) reported, without detailed pathology, a carcinoma of the lesser curvature and pylorus in a bull-terrier. The only case specified by Nieberle and Cohrs is described as a "colloid" cancer. Ball and Chartrain (1931) described the clinical and pathological features of a case of perforated ulcer in the lesser curvature of the stomach of a dog aged nine months. They subjected a nodule on one edge of the ulcer to histological examination and illustrated glands extending into the muscularis mucosae, but it seems not unlikely that these structures were normal transitional glands of the pyloric region.

Of sixty-six carcinomata of dogs studied pathologically over a period of six years in this School, two originated in the gastric mucosa. Unfortunately, neither of the affected dogs could be completely studied and examinations were limited to portions of stomach and of lung in one case and to the stomach and various other abdominal tissues in the other. The first case was an adenocarcinoma of the cardiac portion of the stomach which gave rise to pulmonary metastases. The pyloric mucous membrane was the probable source of the second, a diffuse scirrhous carcinoma identical with that which produces "leather bottle" stomach in man.

RESULTS

Case 1

Subject

A female terrier, 8 years old.

Clinical History

Signs of ill-health were apparent for about a month before the animal was destroyed and comprised at first a cough which was succeeded by retching. At this time appetite was good. Later the cough ceased but vomiting became persistent and appetite was lost. Towards the end gastric tympany developed.

Post-Mortem Examination

A firm thickening about two inches in diameter and accompanied by ulceration was found in the gastric wall near the cardia. The omentum adhered to the serosal surface of the affected area. The lungs showed dull purplish mottling and were largely consolidated, while thickening of the visceral pleura which was greyish-white in appearance, had occurred.

Portions of stomach and lung were submitted for histological examination.

Histopathology

On the mucosal surface of the affected part of the stomach, surviving epithelium was found to be arranged principally in small, densely packed alveoli most of which were lined by a single layer of cuboidal or flattened cells, but other cells did not show any glandular organisation. An extremely scanty stroma, infiltrated by histiocytes and polymorphonuclear leucocytes, separated some of the alveoli. The neoplastic cells had overgrown the lamina propria, penetrated the muscularis mucosae and infiltrated as far as the serous coat. In the submucosa especially the carcinoma showed great pleomorphism and was variously represented by large and small irregular alveoli, some of which contained mucinoid secretion, by thin cords of cells and by isolated single cells. Infiltration and resultant distension of lymphatics had occurred and numerous discrete and aggregated cells were seen within the vessels (Fig. 1). The muscular layers were infiltrated and their fibres extensively separated by the cells. Many of the tumour cells contained mitotic nuclei.

The extreme malignancy of this neoplasm was further illustrated by its ramifications in the lung where there were very numerous areas of tumour growth that varied in character from adenoid clusters to groups of a few cells. The lymphatic vessels of the subpleural and peribronchial zones were heavily infiltrated (Fig. 2), and some connective-tissue hyperplasia had occurred. Many pulmonary alveoli and numbers of blood vessels also contained carcinomatous cells.

Case 2

Subject

A male Scottish terrier, between 3 and 4 years old.

Clinical History

There had been frequent vomiting with a progressive loss of condition over several weeks which culminated in the almost immediate return of practically all solids and fluids taken. The animal remained relatively bright although on one occasion a temperature of 105° F. was noted. Prior to destruction jaundice developed.

Post-Mortem Examination

The greater part of the gastric wall, especially that of the lesser curvature and posteriorly, was thickened to as much as two centimetres by dense greyish-white tissue. There was an irregular ulcer with a firm grey base on the posterior surface of the pyloric membrane 4.5 cm. from the gastro-duodenal junction. Most of the area of the lesser curvature was filled with pale, fawn-grey tissue firmly adherent to the gastric wall, pancreas and omentum. The tumour, which was somewhat nodular, also formed permeating lines over the serosal surface of the stomach, mainly on the lower and posterior surfaces. It had infiltrated the gastro-splenic ligament, the gastric lymph nodes and the left lobe of the pancreas. Lesions were also present in the adrenal.

Histopathology

Examination of various areas of the thickened gastric wall revealed widespread and deep carcinomatous infiltration (Fig. 3). The epithelial cells were pleomorphic and included a proportion of large, irregular cells, some of them containing multiple nuclei. Most of the epithelial elements, however, were moderate in size and ovoid: they were scattered in loose cords as single cells or small aggregates. Their cytoplasm was faintly basophilic. Mitoses, some of them extremely aberrant, were frequently seen. A small minority of the cells were signet-ring type; otherwise their glandular origin was suggested by a small number of small acini (Fig. 4). No layer of the gastric wall had escaped their spread which also involved blood and lymphatic vessels. The submucosa was oedematous. Everywhere, the epithelial hyperplasia was accompanied by a stromal reaction that reached the greatest density in the submucosa, the subserosa and the omentum and lymph nodes, but which was absent from the splenic and adrenal lesions. The deep muscular layers of the stomach suffered greatly from the tumour. Their interfascicular framework was thickened and the myofibrils showed signs of disruption and degeneration. Fibrosis and infiltration were also features of the omentum, the blood vessels and lymphatics which, in common with those of the gastric and diaphragmatic subserosal tissues were seriously involved. The gastric lymph nodes were almost completely replaced by neoplasm. The epithelial cells

were relatively more numerous than in the stomach and sometimes formed substantial but loosely arranged masses, especially in the capsular and subcapsular lymphatics, while the medullary regions were largely collagenous. Quite numerous signet-ring cells and occasional pseudo-acini were seen. The spleen contained small, but widely scattered deposits devoid of stroma. Most of them lay in the sinuses but the splenic veins also showed involvement. A metastatic focus notable for its nuclear distortions was found in the cortex of an adrenal.

SUMMARY

From a survey of veterinary literature, a total of nine confirmed or probable cases of primary gastric carcinoma in dogs has been compiled. Two further cases, an adeno-carcinoma of the cardiac portion of the stomach and a diffuse scirrhous cancer are described. Attention is drawn to the great disparity in the incidence of gastric cancer in man and in the dog.

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FIG. 1

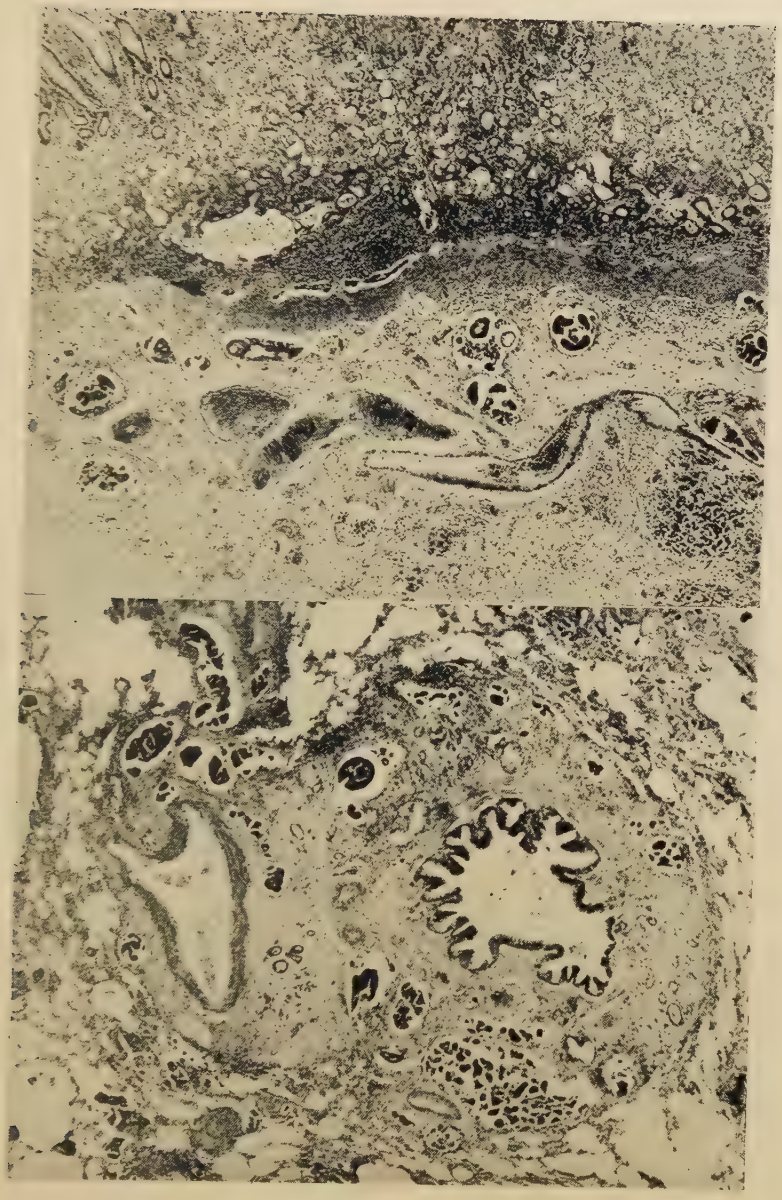

FIG. 2

FIG. 1. *(Case I)*—Stomach. Tumour tissue from the disorganised mucosa has penetrated the muscularis to reach the submucous layer. Many lymphatic vessels contain cells, some of them acinar in arrangement. Haematoxylin and eosin. × 35.

FIG. 2. *(Case I)*—Lung. Showing extensive infiltration of the peribronchial and perivascular lymphatic vessels. Neoplastic cells are also present in alveoli. Haematoxylin and eosin. × 35.

Fig. 3.

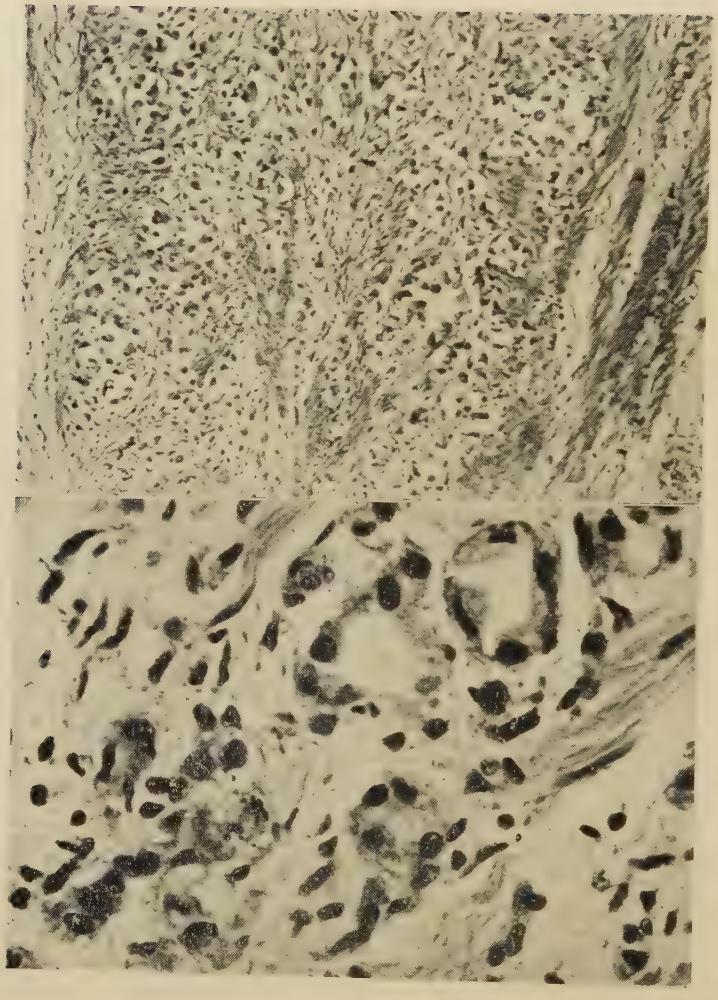

Fig. 4

Fig. 3. (*Case II*)—External muscle coat of stomach. The muscle in the greater part of this area has been destroyed and replaced by rather loosely-knit tumour. Haematoxylin and eosin. × 120.

Fig. 4. (*Case II*)—External muscle coat of stomach. Showing carcinomatous cells in unorganised groups and in the form of small acini (*top right*). Haematoxylin and eosin. × 550.

VARIATIONS IN SERUM PROTEIN LEVELS IN CATTLE*

By

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INTRODUCTION

A recent investigation entailed the comparison of total and differential serum protein levels in two groups of cattle, one consisting solely of bulls aged approximately two years and the other predominantly of heifer calves, heifers and cows of ages varying from six weeks to 15 years. The two groups had been kept under totally different conditions of feeding. It was therefore necessary to study the effects of age, sex and the plane of nutrition on serum protein levels in normal healthy stock. At the same time changes in serum proteins in cases of naturally occurring and experimentally produced liver disease were studied.

METHODS

Normal Cattle. Cattle from three sources were used in the investigation : (*a*) from the stock maintained at the Veterinary Research Laboratory, Vom, for experimental purposes; (*b*) from clinically normal animals from various nomadic Fulani herds slaughtered for human consumption at Bukuru slaughter slab; and (*c*) from the dairy herd at the Livestock Improvement Centre (L.I.C.), Vom. The great majority were of the white Fulani type.

The feeding standards for these cattle were as described in a previous communication (Garner, 1950a). The principal difference between the L.I.C. cattle and the other groups was that the former received full protein supplements from birth.

Most of the animals were examined in September and October, 1949.

Clinical material. Serum protein determinations and histological examinations were made on blood and liver specimens respectively, obtained : (*a*) at random from animals slaughtered for human consumption; (*b*) from cattle which had been experimentally infected with *Trypanosoma congolense* and/or *vivax;* and (c) from cattle which had been given single or repeated subcutaneous injections of carbon tetrachloride. Throughout the work it was endeavoured to use only animals more than five years old in order to eliminate variations due to age (*vide infra*).

Of 184 cattle slaughtered at Vom and Bukuru, 23 showed definite indications of hepatic disease on histological examination. Details are given in Table I.

The general picture in trypanosomiasis was found to be as described by Fiennes, Jones and Laws (1946) and by Fiennes (1950), the principal changes being widespread fatty degeneration and periportal round-cell infiltration accompanied by a varying degree of necrosis of the paren-

*This and the subsequent papers formed part of a thesis submitted for the Fellowship Diploma of the Royal College of Veterinary Surgeons.

TABLE I

TYPES OF LIVER DAMAGE ENCOUNTERED IN CATTLE SLAUGHTERED FOR HUMAN CONSUMPTION

Lesion	*No. of cases*
Portal cirrhosis (diffuse hepatic fibrosis)	9
Focal necrosis	5
Centrilobular necrosis	3
Cholangitis with obstruction of bile ducts	2
Dense periportal mononuclear infiltration (Trypanosomiasis ?)	2
Generalized Fatty change	1
Focal epithelioid foci with giant cells (Tuberculosis ?)	1

chyma cells. *T. vivax* appears to produce a much greater degree of mononuclear infiltration than *T. congolense*.

Single subcutaneous doses of 2, 10 or 30 ml. of carbon tetrachloride produced the typical centrilobular necrosis involving every lobule throughout the organ described in other species (Calder, 1942). The severity of the damage was much the same whatever the dosage, 2 ml. having as great an effect as 30 ml. This is consistent with findings in other animals (Cameron and Karunaratne, 1936; Himsworth, 1947). Carbon tetrachloride given subcutaneously in 10 or 30 ml. doses on alternate days for 15 weeks led to a massive diffuse hepatic fibrosis. The severity of this lesion varied to some degree from animal to animal, but, in general, fibrosis was so extensive as to render it difficult to cut the liver with a knife. Post-necrotic scarring was not encountered.

As only a relatively small number of cases of liver disease were available, a fairly broad classification had to be adopted in attempting to correlate changes in serum proteins with the histological appearance. Lesions were divided into "patchy" and "diffuse" types and these were further subdivided according to the severity of the lesion (Table II).

TABLE II

CLASSIFICATION OF LIVER LESIONS.

1. *Diffuse lesions :*
   - (*a*) Mild ... ... Diffuse hepatic fibrosis (6 cases).
     Centrilobular necrosis (including that due to single injections of carbon tetrachloride) (22 cases).
     Mild "trypanosomiasis lesion" (9 cases).
   - (*b*) Severe ... ... Diffuse hepatic fibrosis (including that due to repeated doses of carbon tetrachloride) (7 cases).
     Severe "trypanosomiasis lesion" (12 cases).
     Generalized fatty change (1 case).
2. *Patchy lesions :*
   - (*a*) Mild ... ... Few areas of hepatic necrosis (4 cases).
   - (*b*) Severe ... ... Large areas of liver cell necrosis (3 cases).
3. *Lesions associated with bile duct obstruction* (2 cases).

Biochemical methods. Blood samples were taken from the jugular vein with the minimum of disturbance to the animal. Total protein and albumin were determined in serum, separated by centrifugation after clotting, by the tyrosine method of Greenberg (1929). Globulin was precipitated by half saturation with ammonium sulphate and the resulting supernatant taken, after centrifugation, for determination of albumin. Globulin was obtained by difference.

In an earlier communication (Garner, 1950b) the conversion factors given for human serum were used, these being the only figures available at that time. Although not invalidating the comparison of results then made, these factors were not strictly applicable to cattle and the opportunity has been taken to represent the data in greater detail using the following equivalents to convert tyrosine to bovine serum albumin and total protein (Snell and Snell, 1937; Garner, 1952): 1 mg. tyrosine $\equiv$ 19·2 mg. albumin $\equiv$ 19·7 mg. total protein.

NORMAL VALUES IN ADULT CATTLE

Histograms showing the frequency distribution of serum total protein, albumin and globulin in 54 healthy adult female stock over 5 years old, on an adequate protein diet are given in Fig. 1. The appropriate frequency curves have been fitted in each case. Total protein shows a positively skewed distribution which is better fitted by a skewed normal than by a logarithmic normal curve; albumin shows a normal distribution, while the globulin diagram is well fitted by a negatively skewed normal curve. With the exception of total protein, the distributions resemble those given for man (Wootton, King, Maclean Smith and Haslam, 1951); in man, total protein shows a normal distribution.

The serum total protein levels of all groups of animals examined are significantly higher than those recorded by Jones (1943) for a mixed group of Ankole and Zebu cattle of both sexes (Range, 7·9 – 9·0; mean 8·3±0·36 for 17 animals). The albumin: globulin ratio (approximately 44: 56) of L.I.C. cattle is in general agreement with results published by other workers also using "salting-out" techniques (Brooksby, 1947).

INFLUENCE OF VARIOUS FACTORS ON PROTEIN LEVELS

Age.

It is well known that protein values for the human infant are lower than those found in the adult, the difference being due to lower values for both the albumin and globulin fractions. Adult levels are reached by the age of 18 months.

Fig. 2 shows the distribution with age of the total protein, albumin and globulin of serum of 126 heifer calves, heifers and cows from the L.I.C., of ages varying from six weeks to 14 years 7 months.

Cattle appear to differ from man in that the increase in the total protein with age is associated with an increase in the globulin fraction only; analysis of the data reveals, in fact, that the albumin

FIG. 1.

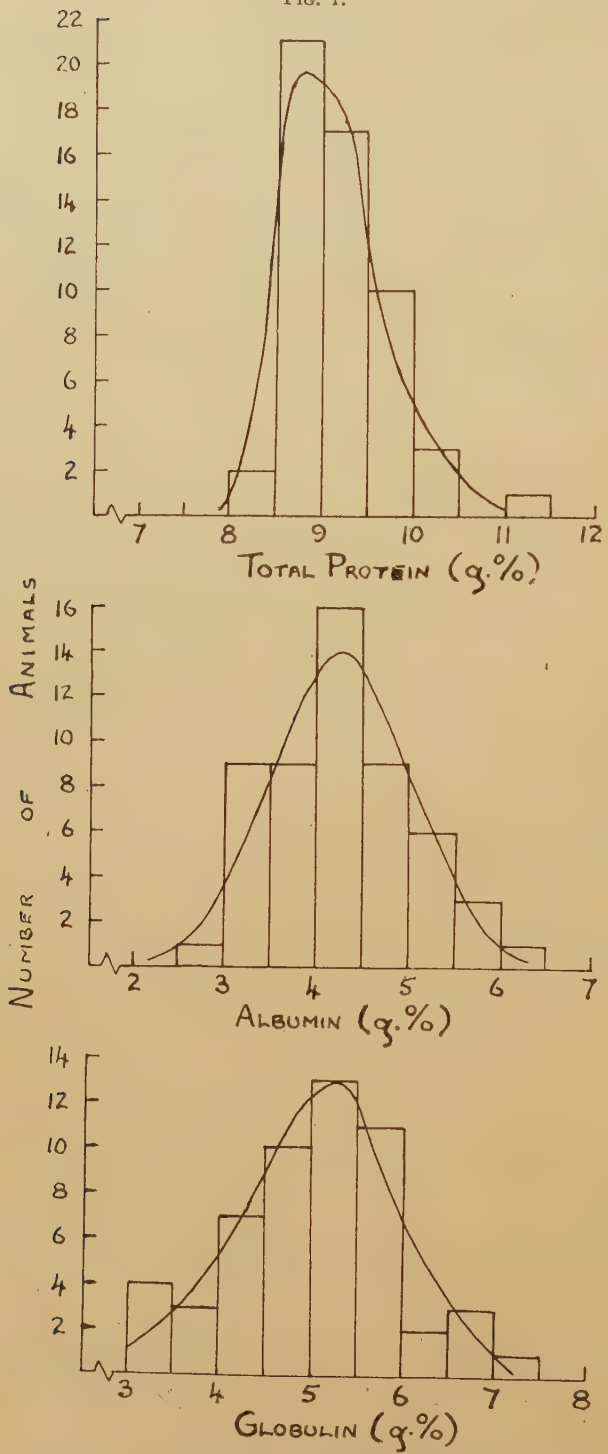

FIG. 1. Distribution of serum total protein, albumin and globulin in healthy adult cows.

FIG. II.

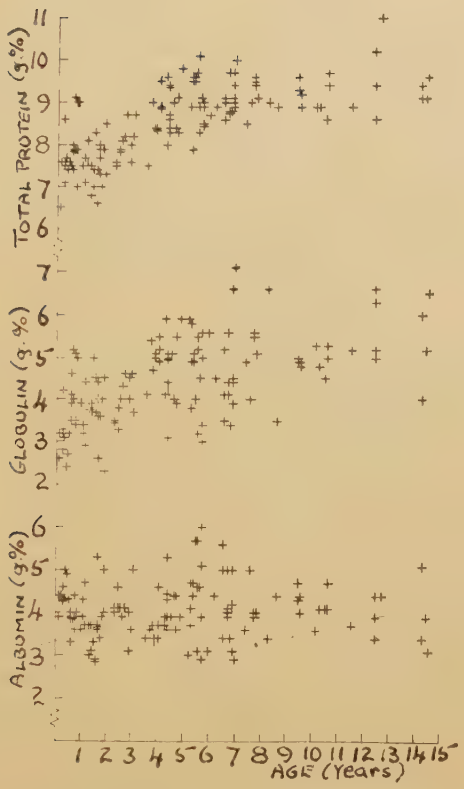

FIG. 2. Variations in total and differential serum proteins with age.

level gradually decreases throughout the life of the animal. A diminution in the rate of increase of total protein and globulin is observed at the relatively late age of approximately five years. This figure can be arrived at by fitting regression lines to the data covering the periods 0 to 5 years and 6 to 15 years and solving the equations relating protein concentration with age thus obtained; nevertheless a steady increase in both constituents persists until at least 15 years.

Sex.

Edwards (1944) implies that there may be a sex difference in serum proteins in cattle. Using the tyrosine method, he found "the total protein content to vary between 7·5 and 9·5 g. per cent, the higher values being obtained in bullocks."

Total and differential protein values for male and female stock maintained (*a*) under range conditions (Bukuru cattle) and (*b*) under conditions of adequate protein nutrition (L.I.C. cattle) are

TABLE III

	Total Protein (*g. per cent*)	*Albumin* (*g. per cent*)	*Globulin* (*g. per cent*)
	Range *Mean ± S.D.*	*Range* *Mean ± S.D.*	*Range* *Mean ± S.D.*
Bukuru Cattle :			
Bulls (79)	6·9 – 11·8	1·9 – 5·3	4·1 – 9·0
	9·25 ± 0·977	3·48 ± 0·650	5·80 ± 1·089
Cows (18)	6·8 – 10·8	2·4 – 5·0	4·4 – 8·0
	9·51 ± 0·982	3·49 ± 0·686	6·04 ± 1·090
L.I.C. Cattle :			
Bulls (10)	8·9 – 10·4	3·3 – 5·1	4·0 – 6·5
	9·72 ± 0·527	4·43 ± 0·724	5·29 ± 0 987
Cows (54)	8·0 – 11·0	2·9 – 6·0	3·0 – 7·1
	9·15 ± 0·52	4·25 ± 0·746	4·97 ± 0·909

shown in Table III. Although in the case of Bukuru cattle, the differences between the values obtained for male and female stock are not statistically significant, the mean total protein level for L.I.C. bulls is significantly greater than that for the corresponding cows ($0{\cdot}01 > P > 0{\cdot}001$), both albumin and globulin contributing to this difference.

TABLE IV

TOTAL AND DIFFERENTIAL SERUM PROTEIN VALUES IN HEALTHY AND DISEASED CATTLE.

No. of Animals	*Total Protein* (*g. per cent*)	*Albumin* (*g. per cent*)	*Globulin* (*g. per cent*)
	Range (*Mean ± S.D.*)	*Range* (*Mean ± S.D.*)	*Range* (*Mean ± S.D.*)
Normal animals :			
161	7·3 – 13·5	2·2 – 5·0	4·2 – 10·7
	(9·4 ± 0·98)	(3·6 ± 0·03)	(5·9 ± 1·16)
Diffuse hepatic lesions :			
Mild :			
37	6·0 – 10·1	2·3 – 4·2	2·1 – 7·2
	(7·8 ± 1·04)	(3·1 ± 0·57)	(4·7 ± 1·09)
Severe :			
20	4·9 – 11·3	1·3 – 4·6	2·6 – 7·8
	(8·1 ± 1·71)	(3·1 ± 0·90)	(4·9 ± 1·31)
Patchy hepatic lesions :			
Mild :			
4	7·5 – 11·7	2·5 – 4·9	4·9 – 6·8
	(9·3 ± 1·51)	(3·7 ± 0·92)	(5·6 ± 0·77)
Severe :			
2	9·7 – 10·0	2·9 – 3·6	6·1 – 7·1
Biliary obstruction :			
2	8·2 – 8·4	3·6 – 3·9	4·3 – 4·8

Plane of nutrition.

If the results for Bukuru and L.I.C. cattle given in Table III are compared it is found that, although the figures for total protein are not widely different, the mean albumin concentration in L.I.C. bulls and cows is significantly greater ($P<0.001$ for both males and females), and the mean globulin concentration significantly less ($0.01>P>0.001$ for males; $P<0.001$ for females) than the corresponding figures for Bukuru bulls and cows respectively. Adult laboratory cattle possess serum protein levels resembling those of Bukuru males and can be considered to be derived from the same population (in the statistical sense).

Liver disease.

Figures obtained for the various classes of liver lesions are given in Table IV. The expression "normal animals" refers to cattle in which liver lesions were either absent or considered to be too slight to be of significance. The differences from "normal" recorded in Table IV are statistically insignificant.

DISCUSSION

It may be inferred from the results given in Table III that Fulani herds and laboratory stock kept under range conditions do not receive amounts of dietary protein sufficient to maintain the serum protein concentration at the normal levels as indicated by the figures obtained for L.I.C. cattle. If L.I.C. cattle are considered to represent "normal" stock, a sex difference can be said to occur, males having rather higher serum protein levels than females.

Alterations in serum proteins of the type recorded in Bukuru and laboratory cattle, *i.e.* an increase in serum globulin with a coincident fall in serum albumin, are commonly associated not only with an inadequate intake of dietary protein, but also with liver disease. As mentioned above, little evidence of wide-spread hepatic disease was obtained.

There is little doubt, on the evidence already outlined, that all the animals with naturally occurring or experimentally produced liver damage were not receiving adequate dietary protein and therefore further changes in serum proteins due to hepatic dysfunction may have been masked. However, the results do seem to indicate a tendency for the serum albumin level to fall, as in man, in severe diffuse hepatic disease (Table IV). A considerable reduction in serum albumin has also been recorded in association with a number of febrile conditions in many animals, including the bovine (Polson, 1951). In man, the total serum globulin frequently remains unaltered owing to the compensating effect of the fall in the α- and β-globulin fractions on the rise in γ-globulin (Maclagan, 1948). In cattle, the general trend appears to be

towards a diminution in total serum globulin and this is supported by the results of flocculation tests. These will be dealt with in a further paper.

SUMMARY

Ranges are given for total and differential protein concentrations in the serum of 54 healthy, well-fed, Zebu cows.

Serum total protein was found to increase with age, fairly rapidly for the first five years of life and then more slowly. This rise was due to an overall increase in serum globulin.

In an adequately fed herd, bulls were found to have significantly higher total and differential serum protein levels than cows.

Cattle kept under range conditions were believed to receive an inadequate protein intake. This was reflected by an increase in serum globulin and a fall in serum albumin.

No definite alterations in serum proteins were observed in cattle with liver disease, although the results presented indicated a tendency for both the globulin and albumin fractions to decrease. It is believed that changes due to a deficiency of dietary protein may mask less marked alterations due to liver disease.

ACKNOWLEDGMENTS.

I wish to thank Mr. S. A. Jonah, of the Veterinary Research Laboratory, Vom and Mallam Yakouba Gana, Veterinary Assistant. Bukuru, for their help in collecting blood and liver samples. I am also indebted to Mr. H. Platt of the Department of Veterinary Pathology, Liverpool University, for making the histological examinations.

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SERUM ALKALINE PHOSPHATASE IN CATTLE IN HEALTH AND DISEASE

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INTRODUCTION

Normal blood serum contains a phosphatase (phosphomonoesterase) classified by Folley and Kay (1936) as Class A1 (pH optimum about 9). The physiological significance of this and of other phosphomonoesterases has been comprehensively reviewed by Moog (1946). The alkaline phosphatase of serum has been found to be elevated in obstructive jaundice (Roberts, 1933; Armstrong, King and Harris, 1934); this increase is of practical diagnostic value in man.

In connection with a general programme of work on liver function tests in cattle, the variation in serum alkaline phosphatase in healthy animals and in animals with liver damage has been studied.

METHODS

Cattle. All animals used were adult Zebu type cattle from the herd maintained at the Veterinary Research Laboratory, Vom, N. Nigeria for experimental purposes, from the dairy herd at the Livestock Improvement Centre, Vom, or from various local Fulani herds slaughtered for meat either at Vom or at Bukuru.

Determination of alkaline phosphatase. All determinations were performed on serum, separated by centrifugation after clotting within 20 to 24 hours after taking the blood sample. The method used was that of King and Armstrong (1934) involving incubation of the serum with a suitable substrate (disodium phenyl phosphate) for 15 minutes at pH 9·0 at 37° C., followed by determination of the liberated phenol with the Folin and Ciocalteu reagent. Readings were made in the Hilger-Spekker photoelectric absorptiometer.

RESULTS

Serum Alkaline Phosphatase in Normal Cattle

The range of values for 294 cattle aged 2 years and upwards has been found to be 4·7 to 62·4 King-Armstrong units per cent., with a median value of 11·4 units. As the accompanying figure shows, the frequency distribution is markedly positively skewed and a logarithmic normal curve fits the data quite well (Fig. 1). On the basis of this fitted curve it is possible to calculate the mean and standard deviation in the usual way and hence to deduce a "normal range". These values have been found to be:

Mean—11·8 units

Normal range (Mean±2 S.D.)—5·8-24·2 units.

FIG 1.

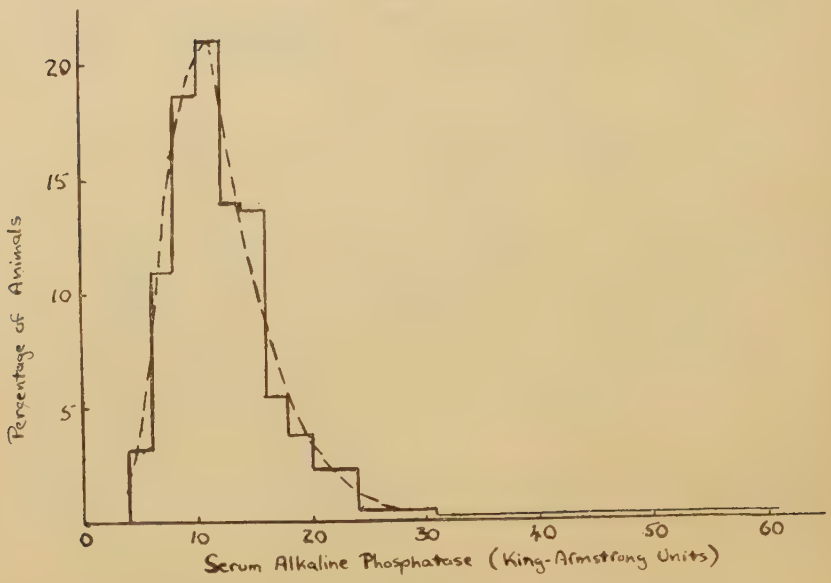

FIG. 1. Distribution of serum alkaline phosphatase in 294 Zebu cattle. The broken line shows a logarithmic normal curve fitted to the data.

Serum Alkaline Phosphatase in Liver Disease

As mentioned above, determination of serum alkaline phosphatase is a valuable aid to the differential diagnosis of obstructive jaundice in man. So far as is known, changes in cattle with liver disease have not been studied. Results obtained in a number of cases of liver disease (for method of classification see Garner, 1952) are listed in Table I. The mean value recorded for cases of severe diffuse hepatic disease is significantly higher (assuming a logarithmic normal distribution in these cases) than normal ($P<0.001$).

TABLE I
ALKALINE PHOSPHATASE IN CATTLE SERUM IN LIVER DISEASE.

	No. of animals	*Alkaline Phosphatase (King-Armstrong Units/100 ml.)*	
		Range	*Mean*
Normal animals	294	4·7 – 62·4	11·8
Diffuse lesions :			
Mild	37	7·8 – 30·2	13·2
Severe... ...	20	8·2 – 46·5	17·1
Patchy lesions :			
Mild	4	4·7 – 16·3	10·6
Severe... ...	3	13·1 – 29·5	16·6
Biliary obstruction : ...	2	10·3 – 15·2	12·7

Mode of Excretion of Alkaline Phosphatase in Cattle

Cantarow, Stewart and McCool (1936) have found that ligation of the common bile duct in the cat fails to produce an increase in serum alkaline phosphatase. According to Flood, Gutman and Gutman (1937), the cat's kidney is able to clear alkaline phosphatase and, in this animal, excess enzyme is apparently excreted in this way.

TABLE II

ALKALINE PHOSPHATASE CONTENT OF HUMAN AND CATTLE URINE

	No. of samples	*Alkaline Phosphatase (King-Armstrong units/100 ml.)*	
		Range	*Mean*
Human urine	18	0 – 0·32	0·08
Cattle urine	15	0 – 0·38	0·06

TABLE III

ALKALINE PHOSPHATASE CONTENT OF HUMAN AND OX GALL-BLADDER BILE.

	Alkaline Phosphatase (King-Armstrong Units/100 ml.)		
	Mean		*S. D.*
Human bile (15 samples obtained 12 to 24 hours after death)	14·5	±	7·41
Cattle bile (21 samples obtained a few minutes after death)	4·2	±	1·24

There is no evidence that alkaline phosphatase is normally excreted by the kidney in cattle, as the urine contains amounts comparable with those in human urine (Table II). These small amounts are presumably derived from the renal tubules. Alkaline phosphatase is present in cattle bile, although the concentration is somewhat lower than that in human bile (Table III). The higher values encountered in human bile may be a result of changes in composition due to stasis in the gall-bladder or, more probably, may simply indicate, in accordance with the findings of Schmidt and Ivy (1937), that the gall-bladder of the cow has little or no concentrating capacity.

DISCUSSION

Allcroft and Folley (1941) and Duckworth and Godden (1943) give values for serum alkaline phosphatase in cows and calves of

0·3 to 114·3 King-Armstrong units and 3·4 to 13·0 Bodansky units (approximately equivalent to 8 to 35 King-Armstrong units) per cent. respectively. The overall range in Nigerian cattle falls between these two groups.

The frequency distribution for Zebu cattle, however, does not follow that of Allcroft and Folley's for either dairy or Hereford cows. This apparent discrepancy may be due to a breed difference. Allcroft and Folley report a diminution in the serum alkaline phosphatase concentration with age. It is unlikely that a difference in average age of the respective groups can account for the raised mean values in Zebus, as the data available indicate that there is little variation after the age of 2 years.

The values for serum alkaline phosphatase in cattle are, in general, very high compared with most other species, although relatively high values have been encountered in dogs (King and Armstrong, 1934) and rats (Gould, 1944). Allcroft and Folley (*loc. cit.*) reject the suggestion that the high values in cattle are due to the presence of an enzyme activator and conclude that there are species differences with regard to normal values and changes in serum phosphatase.

Although there is a significant increase in serum alkaline phosphatase concentration in severe generalized liver disease, the wide range observed in normal cattle precludes determination of the enzyme as a reliable diagnostic aid in liver disease. The results in cases showing definite histological signs of biliary obstruction were surprising. Two attempts were made to produce experimental obstructive jaundice in calves by ligation of the common bile duct. No changes in serum alkaline phosphatase were observed and it was later found that one operation certainly had been unsuccessful, and there were doubts about the other.

Since it appears that the bile is the principal channel of excretion of alkaline phosphatase in cattle as in most other species, it must be concluded that obstruction of the bile ducts, in the cases encountered, was incomplete.

SUMMARY

The normal range of serum alkaline phosphatase in 294 Zebu cattle over 2 years old has been found to be 5·8 to 24·2 King-Armstrong units, with a mean value of 11·8 units.

Although significant deviations from normal were encountered in cases of severe diffuse liver damage, the wide range in normal animals prohibits the use of serum alkaline phosphatase as an indicator of hepatic dysfunction.

The main channel of excretion is believed to be *via* the bile and the failure to obtain an increase in serum alkaline phosphatase in obstructive jaundice is considered to be due to incomplete occlusion of the biliary passages.

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DISTURBANCES IN CARBOHYDRATE METABOLISM IN CATTLE ASSOCIATED WITH LIVER DISEASE

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INTRODUCTION

One of the most important functions of the liver is its capacity to form and store glycogen. In hepatic disease the ability of the organ to convert precursors into glycogen may be reduced and its glycogen content, in consequence, depleted.

The glycogenetic capacity of the liver may be estimated by administration of some precursor orally, subcutaneously or intravenously followed by measurement of the rate of disappearance of this substance from the blood. Glucose, fructose, sucrose, galactose, dihydroxyacetone and lactic acid are all converted to glycogen by the liver and all have been used in human work for the performance of "tolerance" tests.

The glycogen storage capacity of a diseased liver may be less than normal. It is possible to obtain an estimate of the glycogen content of the liver directly on biopsy samples; such specimens, however, may not be representative of the whole organ. Indirectly, the glycogen reserve of the whole liver is said to be measured by the rise in blood sugar following injections of a standard dose of adrenalin (Loeper and Verpy, 1917; Kinsell, Michaels, Weiss and Barton, 1949) or by the degree of sugar excretion, hypoglycaemia and ketosis provoked by phlorizin administration (Mirsky and Nelson, 1944). The validity of the response to adrenalin as a measure of the glycogen storage capacity of the liver will be discussed later.

THE GALACTOSE TOLERANCE TEST

Galactose has been preferred in the present work as it cannot be utilized by the tissues until after conversion to glucose by the liver.

The intravenous test developed by King and Aitken (1940), has been used throughout. Galactose was given intravenously in the form of a 50 per cent. solution at the rate of 0·5 g./kg. body weight to animals fasted overnight. Immediately prior to and $\frac{1}{2}$, 1, $1\frac{1}{2}$, and 2 hours after the injection, 0·05 ml. of blood was pipetted directly from a needle inserted into the jugular vein into an isotonic sodium sulphate-sodium tungstate solution. Galactose was determined by the Harding-Nelson method described by King and Garner (1947) after removal of the glucose by fermentation with a suspension of washed baker's yeast for 15 minutes at 37° C.

In order to facilitate comparison of results the modified "galactose time" introduced by Sherlock (1946) has been calculated. This expresses the rate of disappearance of galactose from the blood in minutes and is given by

$$\text{Galactose time} = \frac{(a-b)}{ax}$$

where a = 30 minute value for blood galactose, b = last figure for blood galactose before complete elimination from the blood stream (when the two hour sample still contained galactose, the two hour value has been taken), x = 30, 60 or 90 minutes, according to whether b is the 1, $1\frac{1}{2}$ or 2 hour blood galactose value.

RESULTS

The test was carried out on seven clinically normal animals, liver biopsy specimens from which showed no lesions; on four animals showing mild diffuse liver damage due to a single subcutaneous injection of carbon tetrachloride; on five animals in which severe diffuse lesions had been experimentally produced by repeated injections of carbon tetrachloride (Garner, 1952); on a bull which was apparently "normal" but at autopsy, showed

TABLE I

GALACTOSE TIMES IN NORMAL AND DISEASED CATTLE.

Normal animals						
Animal No.	924	938	947	955	1,126	1,175
Galactose time (minutes) ...	160	111	95	142	133	124
			(Mean, 123)			
Mild diffuse liver damage						
Animal No.	952	961	974	1,105		
Galactose time (minutes) ...	97	64	67	94		
			(Mean, 80)			
Severe diffuse liver damage						
Animal No.	932	1,032	1,146	1,151	1,152	
Galactose time (minutes) ...	101	233	194	138	112	
			(Mean, 156)			
Obstructive jaundice			*Haemolytic jaundice :*			
Animal No.	245				210	
Galactose time (minutes) ...	94				90	

cholangitis with obstruction of the bile ducts and on a further animal suffering from haemolytic jaundice as a result of anaplasmosis. All animals were Zebu stock aged two years or more.

Table I lists the galactose times and Fig. 1 shows the rate of removal of galactose from the blood in individual cases.

FIG. 1.

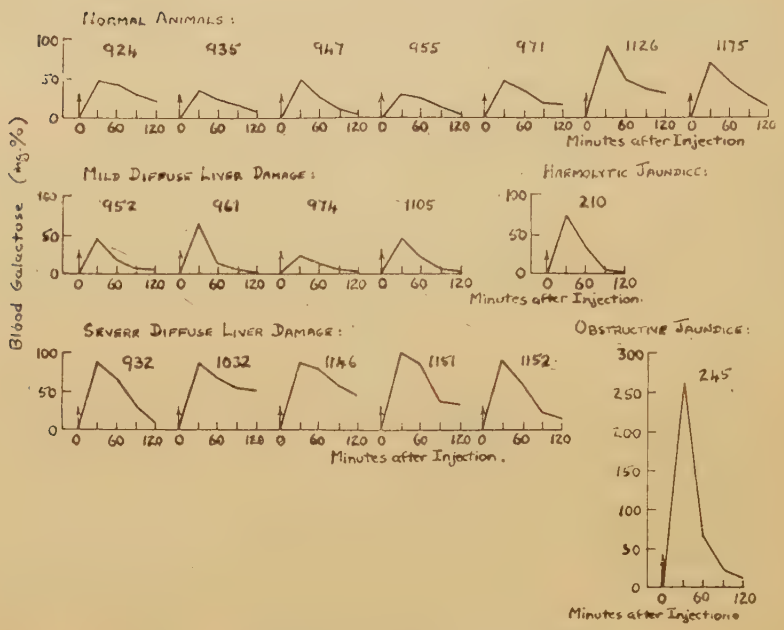

FIG. 1. The galactose tolerance test in Cattle.

Alterations in Blood Glucose following Intravenous Galactose.

The changes in blood glucose and serum inorganic phosphate following intravenous injection of galactose (0·5 g. per kilo) were studied in six apparently normal animals. These observations were made in order to examine the possibility of using the galactose tolerance test as a dual indicator of hepatic and pancreatic dysfunction.

As galactose has not the same reducing power as glucose towards the modified Schaffer-Hartmann-Harding copper reagent (potassium iodate omitted) used in this work, the glucose content of the blood cannot be determined directly by subtracting galactose concentration, determined after fermentation with yeast, from the total reducing sugar. It was found that galactose possessed 72 per cent, of the reducing power of glucose under the conditions of the determination. This figure is in good agreement with the results given by King (1946) who, using the original Harding reagent for the iodometric method, found the reducing powers of galactose and glucose to be in the ration 116: 162. The glucose concentration, in the present instance, is arrived at from the expression:

Glucose concentration = total reducing sugar (expressed as glucose) − 0·72 × galactose concentration (all values in mg. per cent.).

The results are shown in Fig. 2.

FIG. 2.

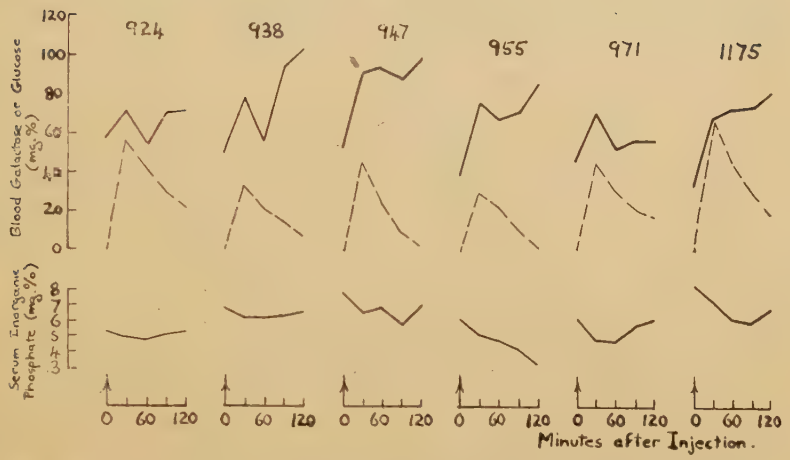

FIG. 2. Changes in blood glucose and serum inorganic phosphate produced by intravenous galactose.

———— Blood glucose.
--------- Blood galactose.

THE ADRENALIN-RESPONSE TEST

Some doubt has been thrown on the validity of the assumption, that the rise in blood glucose following injection of adrenalin is, in fact, indicative of the glycogen content of the liver. It was believed for many years that the rise in blood sugar resulted from accelerated hepatic glycogenolysis, but evidence has accumulated which indicates that adrenalin probably depresses the rate of glucose assimilation (Somogyi, 1950).

If this is so the lowered response to adrenalin associated with liver disease indicates that the liver cells have already lost much of their capacity to take up glucose and convert it to glycogen rather than that they have reduced ability to store glycogen. The adrenalin-response test would then be but another test of the glycogenetic capacity of the liver and the assumption that this test "as contrasted to procedures based on oral or intravenous administration of sugars, supplies unequivocal information, in that the initial elevation of blood sugar in response to adrenalin can relate only to pre-existing hepatic glycogen" (Kinsell *et al.*, 1949), becomes invalid.

The adrenalin-response test, if carried out under suitably standardized conditions is believed by Kinsell *et al.* (*loc. cit.*) to provide a dependable indication of the presence of acute and chronic liver disease in man. Earlier unfavourable reports are believed to be due to lack of adequate preliminary standardization,

Method. 1/100 gr. of adrenalin hydrochloride per 10 kilos body weight (67 μg. per kilo) was injected subcutaneously into cattle fasted for 12 hours and the venous blood sugar determined hourly for 5 hours following the injection. The results are recorded in Fig. 3.

FIG. 3.

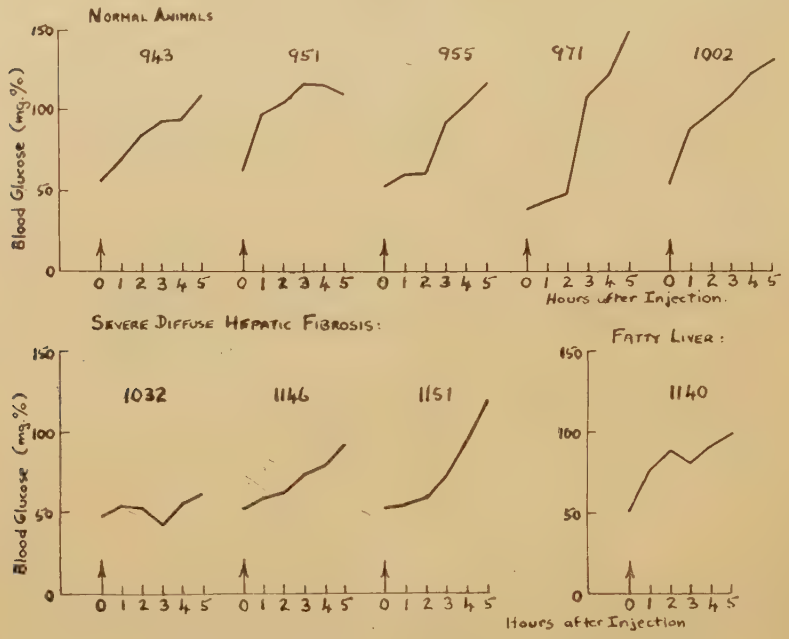

FIGURE 3. The Adrenalin-response test in cattle.

TABLE II

RATES OF INCREASE OF BLOOD GLUCOSE FOLLOWING ADRENALIN INJECTION IN NORMAL AND DISEASED CATTLE.

Normal animals					
Animal No.	943	951	955	971	1,002
Rate of increase (% per hour) ...	21·6	28·4	24·3	64·0	31·9
Severe diffuse hepatic fibrosis :					
Animal No.	1,032	1,146	1,151		
Rate of increase (% per hour) ...	−1·8	14·2	13·8		
Fatty liver (Trypanosoma vivax)					
Animal No.	1,140				
Rate of increase (% per hour) ...	21·8				

In man, the test is reported in terms of the difference between the fasting and maximal post-adrenalin blood sugar. In cattle the blood glucose level was still rising after 5 hours so that the average

percentage increase in blood sugar per hour over the first 3 hours has been calculated from the expression:

$$\text{Rate of increase per hour} = \frac{3 \text{ hour value} - \text{initial value}}{\text{initial value}} \times \frac{100}{3}$$

The values obtained are given in Table II.

DISCUSSION

Galactose persists in the blood for a considerably longer time in normal cattle than in humans. Sherlock (1946) records that in 40 out of 41 patients without liver disease, all galactose had disappeared within two hours. She gives the normal galactose time at 30 to 92 minutes (mean for 41 subjects, 61). Very similar results were obtained by Greenman (1950). If the liver is the only organ responsible for removal of galactose from the blood, as is believed to be the case, the rate of glycogenesis from galactose in cattle must be approximately half that in man.

The reduced galactose time observed in cattle with mild diffuse liver damage is striking and is statistically significant ($0{\cdot}02 > P > 0{\cdot}01$). This finding appears to be directly analogous to that of Sherlock (1946) in mild acute hepatitis in man. The shape of the galactose tolerance curve (Fig. 1) gives a clearer indication of the presence of liver disease than the galactose time. The results resemble closely those obtained by King and Aitken (1940) for human patients.

The fall in serum inorganic phosphate accompanying uptake of galactose (Fig. 2) presumably indicates that glycogen formation from galactose involves a phosphorylation mechanism. An almost identical picture was found by Free and Leonards (1943) following intravenous injection of galactose to normal dogs. The blood glucose, however, responds to intravenous galactose in a manner analogous to that found in the diabetic rather than the normal human (Greenman, 1950). This prolonged rise may be due to slower conversion of galactose, *via* glycogen, to glucose or may point to a low tolerance for glucose in normal adult cattle (see below).

Although there is some difference in the response to adrenalin of normal and diseased cattle in the small series of animals examined, the results are variable and indecisive. The initial rate of increase in blood glucose however, provides a fairly clear distinction between normal and diseased animals (Table II). The test could be more simply carried out using this figure by taking blood samples before and 3 hours after the adrenalin injection.

The rise in blood glucose due to adrenalin is greatly prolonged in cattle as compared with man. Kinsell *et al.* (1949) found that the post-adrenalin blood sugar returned to the pre-injection level within two hours. Admittedly a larger dose was used in the present work than in the human work cited (67 μg. of adrenalin

hydrochloride per kilo as compared with 10 μg. per kilo) but Kinsell does, in fact, state that larger amounts do not significantly increase the maximal response.

The prolonged action of adrenalin in cattle may reflect a reduced rate of absorption from the subcutaneous tissues but, in the light of the effect of galactose on the blood glucose mentioned above, it seems more likely that it is due to a low rate of assimilation of glucose by peripheral tissues in cattle.

McCandless, Woodward and Dye (1948) have found that tolerance to intravenous glucose (1 g./kg.) in the normal non-fasted adult sheep resembles that in the diabetic carnivore, pre-injection levels being reached only after 4 to 6 hours. Reid (1950) has also reported that there is a smaller rate of uptake of glucose by the tissues of the sheep than occurs in non-ruminants. Bell and Jones (1945), on the other hand, using three to four-year-old cattle (males and non-gravid heifers) and intravenous glucose at the rate of 0·1, 0·2 or 0·5 g./kg. found that the blood sugar returned to normal, in all cases, within two hours, irrespective of the dosage. Holmes (1950) found similar results in two pregnant heifers using a dose rate of 0·3 g./kg.

It is difficult to reconcile the results reported here and by McCandless *et al.* (1948) with those of other workers, particularly Bell and Jones. It can be argued *a priori* that, because of the peculiar nature of the products of ruminant digestion, the ruminant might be expected to have a reduced tolerance to glucose. Thus McCandless suggests that the low tolerance to injected glucose is a result of the high rate of gluconeo-genesis from absorbed fatty acids in the non-fasting state. As she found the tolerance to be still further reduced after three days fasting, gluconeogenesis from products of digestion cannot be the only factor responsible. Since propionic acid is the only definite carbohydrate precursor available to the ruminant, this type of animal can probably be considered as existing on a high fat, moderate protein, low carbohydrate diet. It is well known that tolerance to ingested glucose is reduced considerably in non-ruminants if the carbohydrate of the diet is reduced and/or replaced by protein or fat, but particularly by a high fat diet (Peters and Van Slyke, 1946). A lowered rate of glucose utilization in ruminants is therefore not surprising.

Whatever the mechanisms underlying the prolonged response to adrenalin and the delayed removal of galactose and glucose from the blood, it is evident that neither the adrenalin-response test nor the galactose tolerance test, at least in the form presented here, are capable of an unequivocal interpretation. Nevertheless, of the liver function tests in general use in human clinical medicine which have been applied to cattle, tests involving the estimation of the ability of the liver to synthesize glycogen appear to have more promise than most. The galactose test is, unfor-

tunately, expensive and time-consuming, but has given the most encouraging results so far.

As the most important normal precursor of glycogen in ruminants appears to be propionic acid, it is possible that a propionate tolerance test might provide a more sensitive and more physiological approach to the detection of liver damage in cattle. Preliminary enquiries along these lines have been made by Australian workers (Jarrett, 1951).

SUMMARY

The rate of removal of intravenously injected galactose from the blood of normal cattle and cattle with experimentally produced liver damage has been studied. The rate of glycogenesis from galactose in cattle appears to be about half that in man.

Intravenous galactose stimulates an increase in the blood glucose level in normal cattle similar to that seen in the human diabetic. The significance of this and other relevant findings is discussed in the light of present knowledge of intermediary metabolism in ruminants.

The validity of the adrenalin-response test as an indicator of the glycogen storage capacity of the liver is questioned. The application of this test to cattle is described.

It is concluded that the galactose tolerance test and the adrenalin-response test, with suitable modification, could be applied to the detection of hepatic dysfunction in cattle.

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FLOCCULATION TESTS IN THE DETECTION OF LIVER DISEASE IN CATTLE

By

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INTRODUCTION

A large number of tests have been devised for the detection, differential diagnosis and prognosis of liver disease in man which depend for their action on the production of a turbidity, flocculation, precipitation or gel formation on the addition of certain reagents to serum from cases of hepatic disease. These were originally classed as "empirical" tests but it has since become evident that all reflect changes in the albumin: globulin ratio; nearly all give a positive result in the presence of a sufficient increase in the γ-globulin moiety of the serum protein complex.

It is obvious that any test which depends upon an increase in the γ-globulin fraction of serum will give a positive result, not only in cases of liver disease, but also in other conditions (for example, infections) in which this fraction is increased. Nevertheless, flocculation reactions have found an important place in the detection of liver dysfunction in man and recent work indicates that it is possible to correlate certain of these tests with definite histological features. Thus a significant correlation has been observed between diffuse liver cell damage and cephalin-cholesterol and thymol turbidity tests (Franklin, Popper, Steigmann and Kozoll, 1948), between distorted reconstruction of the lobular pattern and these tests and between cell regeneration and the thymol turbidity test (Franklin *et al.*, 1948; Hoffbauer, Evans and Watson, 1945).

The chemical and clinical significance of flocculation tests as applied to human work has been reviewed by Maclagan (1948).

METHODS

Clinical Material.

Sera and liver samples were taken at random from a series of animals slaughtered for human consumption. A description of the lesions encountered and the method of classification has been given elsewhere (Garner, 1952). "Normal" animals are those which showed negligible hepatic changes. It is regretted that the majority of prepared specimens were not of a standard to permit detailed histological examination.

Biochemical Methods.

It is not proposed to give details of the techniques unless modifications of the methods published have been made.

Alcacid Reaction (Waldenström, 1945). This test has not been applied to the detection of hepatic disease, but since it is believed to respond to an increase in serum globulin it has been included. No graduation of the response was attempted, results being recorded simply as negative or positive.

Alcohol Dilution Test (Love and Mawson, 1948). 0·2 ml. of serum were used.

Cadmium Turbidity Test (Wunderly and Wuhrmann, 1945, 1947). As originally described, the relative turbidities were assessed visually and arbitrary values assigned from 0 to 3. It has been found more convenient to compare the results against artificial formazine standards (King, 1946) as in the alcohol dilution and thymol turbidity tests and to assign degrees of turbidity from 0 to 10.

Cephalin-Cholesterol Flocculation Reaction (Hanger, 1939). This was carried out following the procedure of Dick (1945) and using "Difco" cephalin-cholesterol antigen supplied by Messrs. Baird and Tatlock, London, Ltd.

Serum Colloidal Gold Reaction (Maclagan, 1946). Several versions of this test were performed using 0·05 ml. of serum and 0·5 ml. of the original barbitone buffer of pH 7·7 of Maclagan, and 0·05 and 0·1 ml. of serum and 0·5 ml. of the modified buffer of pH 7·55 recommended by Latner and Pendleton (1949).

Formol-Gel Test (Spackmann, 1921). The test described by Napier (1922) for the diagnosis of Kalar-Azar has been used. Results have been classified as negative or positive.

Takata-Ara Test (Takata and Ara, 1926). The procedure described by Kolmer and Boerner (1938) has been followed throughout. According to these authors, a positive reaction is said to result when there is a persistent, dense, felt-like precipitate in at least three tubes, at least one of which should contain serum at a dilution of 1 in 32 or higher. As most of the cattle sera examined gave a positive result according to this interpretation, the test was read by assigning a figure according to the number of tubes in which precipitation occurred, i.e. from 0 to 8.

Ucko's Modification of the Takata-Ara Test (Ucko, 1936, 1942).

Britton's Modification of the Takata-Ara Test (Britton, 1945).

Thymol Turbidity Test (Maclagan, 1944). This test has been applied using 0·05, 0·1 and 0·2 ml. of serum and the thymol buffers of pH 7·8 of Maclagan and of pH 7·55 of Latner and Pendleton (1949).

Thymol Flocculation Test (Maclagan, 1947). This was performed as an adjunct to the thymol turbidity test by allowing the tubes to stand overnight and recording flocculation.

The sensitivity of all reagents used in these tests was checked against sera obtained from a variety of African patients, with hepatic disease in the Sudan United Mission Hospital, Vom, N. Nigeria.

CORRELATION OF FLOCCULATION TESTS WITH CHANGES IN SERUM PROTEINS IN CATTLE.

As mentioned above, the majority of flocculation tests indicate increases in the γ-globulin concentration; a reduction in the albumin fraction is also said to be effective in producing positive results

in certain cases. In order to ascertain whether these tests bear the same relationship to change in serum proteins in cattle as in man, a comparison was made between the results obtained with the various tests described above and (*a*) total serum globulin (in lieu of γ-globulin only) and (*b*) the globulin: albumin ratio. If the correlation coefficient between the test and total globulin was found to have a greater level of significance than that between the test and the globulin: albumin ratio it was concluded that changes in albumin concentration had little, if any, effect on the result of the test and *vice versa*.

TABLE I

LEVELS OF SIGNIFICANCE (P) OF THE CORRELATION COEFFICIENTS BETWEEN (*a*) FLOCCULATION TESTS AND TOTAL SERUM GLOBULIN AND (*b*) FLOCCULATION TESTS AND THE GLOBULIN : ALBUMIN (G/A) RATIO.

Test	*Number of tests*	P *Globulin*	*G/A*
Alcacid	230	<0·01	<0·01
Alcohol dilution	87	<0·01	<0·01
Cadmium turbidity	227	>0·1	<0·01
Serum colloidal gold :			
0·05 ml. serum & buffer pH 7·7	72	>0·1	>0·1
0·05 ml. serum & buffer pH 7·55	136	>0·1	>0·1
0·1 ml. serum & buffer pH 7·55	91	<0·01	>0·1
Formol-gel	227	<0·01	<0·01
Takata-Ara	321	<0·01	<0·01
Britton's modification of the Takata-Ara test ...	151	>0·1	>0·02
Thymol turbidity			
0·05 ml. serum & buffer pH 7·8	72	<0·01	>0·1
0·05 ml. serum & buffer pH 7·55	139	<0·01	>0·1
0·1 ml. serum & buffer pH 7·55	91	<0·01	<0·01
0·2 ml. serum & buffer pH 7·55	91	<0·01	<0·01
Thymol flocculation.			
0·05 ml. serum & buffer pH 7·8	72	>0·02	>0·1
0·05 ml. serum & buffer pH 7·55	139	>0·05	>0·1
0·1 ml. serum & buffer pH 7·55	91	<0·01	<0·01
0·2 ml. serum & buffer pH 7·55	91	<0·01	<0·01

Table I lists the levels of the correlation coefficients obtained.

FLOCCULATION TESTS IN LIVER DISEASE IN CATTLE.

The results obtained in a variety of cases of liver disease are summarised in Table II, which also indicates where correlation has been found to occur between the results of tests and the occurrence of liver disease.

DISCUSSION.

It will be observed that the cephalin-cholesterol reaction and Ucko's modification of the Takata-Ara test, although included under " Methods " are excluded from the Tables. This is because both tests gave consistently positive results. Bonsmann and Wiesner (1951) have recorded that the Takata-Ara test and many of its modifications give positive results with sera from normal

TABLE II

FLOCCULATION TESTS IN CATTLE. (THE FIGURES GIVEN ARE THE MEAN VALUES FOR THE NUMBER OF OBSERVATIONS INDICATED BY THE BRACKETED FIGURE FOLLOWING THEM.)

Normal animals	*Diffuse lesions*				*Patchy lesions*				*Biliary obstruction*	
	Mild		*Severe*		*Mild*		*Severe*			
Alcacid test :										
0·43	(167)	0·14[3]	(35)	0·25 (20)	0·25	(4)	0·50	(2)	0·00	(2)
Alcohol dilution test :										
4·44	(50)	3·88	(17)	4·62 (8)	4·00	(1)	—		—	
Cadmium turbidity test :										
6·40	(161)	6·08	(26)	5·22 (18)	3·75	(4)	6·50	(2)	8·50	(2)
Serum colloidal gold reaction :										
0·05 ml. serum and buffer pH 7·7,										
0·82	(49)	0·22	(9)	0·26 (7)	0·00	(2)	—		—	
0·05 ml. serum and buffer pH 7·55,										
0·78	(112)	1·65[1]	(17)	1·18 (11)	3·50[2]	(2)	0·00	(1)	2·50	(2)
0·1 ml. serum and buffer pH 7·55,										
0·76	(66)	0·25	(4)	0·00 (3)	0·00	(1)	0·50	(2)	—	
Formol-gel test :										
0·32	(159)	0·17	(30)	0·33 (18)	0·50	(4)	1·00[1]	(2)	0·00	(2)
Takata-Ara test :										
4·32	(162)	4·60	(35)	4·60 (20)	4·50	(4)	5·00	(2)	4·50	(2)
Britton's modification of the Takata-Ara test :										
1·65	(100)	3·00[2]	(17)	3·91[4] (11)	1·50	(2)	1·00	(2)	2·00	(2)
Thymol turbidity test :										
0·05 ml. serum and buffer pH 7·8,										
1·84	(62)	1·25	(4)	1·67 (3)	1·00	(1)	0·52	(2)	—	
0·05 ml. serum and buffer pH 7·55,										
1·52	(111)	0·69[1]	(13)	0·60 (10)	0·67	(3)	1·00	(2)	—	
0·1 ml. serum and buffer pH 7·55,										
3·02	(50)	3·00	(13)	2·00 (8)	3·00	(1)	—		4·50	(2)
0·2 ml. serum and buffer pH 7·55.										
4·84	(50)	5·54	(13)	4·50 (8)	4·00	(1)	—		6·00	(2)
Thymol flocculation test :										
0·05 ml. serum and buffer pH 7·8,										
1·34	(62)	1·00	(4)	0·67 (3)	1·00	(1)	0·50	(2)	—	
0·05 ml. serum and buffer pH 7·55,										
1·10	(111)	0·69	(13)	0·80 (10)	0·67	(3)	0·50	(2)	—	
0·1 ml. serum and buffer pH 7·55,										
1·72	(50)	1·77	(13)	1·50 (8)	2·00	(1)	—		2·50	(2)
0·2 ml. serum and buffer pH 7·55,										
2·56	(50)	3·08	(13)	2·25 (8)	3·00	(1)	—		4·00	(2)

[1] Significantly different from normal at the 10% level
[2] ,, ,, ,, ,, ,, ,, 5% ,,
[3] ,, ,, ,, ,, ,, ,, 2% ,,
[4] ,, ,, ,, ,, ,, ,, 1% ,,

animals. Ucko's test could doubtless have been suitably modified by adjusting the concentrations and amounts of reagents used, but as the method had no advantage over Britton's test, it was abandoned.

The cephalin-cholesterol test is said by Schaffer (1949) to yield good results in cattle. The antigen is definitely unstable (Steinberg, 1949) and this may have been a contributory cause of the unsatisfactory results, although what are considered to have been adequate precautions (leaving tubes in the dark in the ice-chest) were taken.

In the majority of tests showing any correlation with changes in serum proteins, analysis of the data reveals that the major factor in bringing about an increased degree of flocculation is an overall increase in the globulin fraction. This is so in the case of the alcacid, alcohol dilution, colloidal gold, formol gel, thymol turbidity and thymol flocculation reactions. In the case of the cadmium turbidity test and the Takata-Ara test, both in its original form and in the form described by Britton, a decrease in serum albumin appears to play a significant part.

The sensitivity of the serum colloidal gold and thymol flocculation tests in detecting changes in serum proteins was found not to be effectively increased by using 0·1 or 0·2 ml. of serum in place of the 0·05 ml. originally recommended; the thymol turbidity test is, however, rendered more sensitive by using 0·1 or 0·2 ml. of serum and a buffer of pH 7·55.

Very few of the tests examined gave any degree of correlation with the existence of liver disease. The only one which appears to be of use in cattle is Britton's modification of the Takata-Ara test. Using this test, the serum from diseased cattle gives an increased degree of flocculation. A point decidedly in favour of this test is that a positive result should give a fairly specific indication of the occurrence of liver disease as changes in serum proteins, which might be due to many causes, are much less significantly correlated (Table I).

The alcacid test gives a lower degree of flocculation in certain cases of liver disease and the general tendency appears to be for these tests correlated with serum globulin (and relatively unaffected by changes in serum albumin) to give reduced flocculation, while those showing a greater correlation with changes in the globulin : albumin ratio exhibit enhanced flocculation.

The results serve to strengthen the hypothesis already put forward (Garner, 1952) that liver disease in cattle is associated with a fall in both the albumin and total globulin of the serum. Electrophoretic examination of cattle sera would serve to confirm this.

SUMMARY

A number of flocculation tests have been applied to the detection of liver disease in cattle.

The majority of these tests were found to reflect changes in serum proteins in cattle as in man.

It is considered that Britton's modification of the Takata-Ara test could serve as a useful indicator of the existence of hepatic disease in cattle.

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DYE EXCRETION TESTS IN THE DETECTION OF LIVER DISEASE IN CATTLE

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INTRODUCTION

Advantage has been taken of the fact that certain water-soluble dyes are excreted in the bile, to devise tests of the excretory capacity of the liver. The principal dyes used have been phenoltetrachlorophthalein, phenoltetraiodophthalein (iso-iodeikon), phenoltetrabromophthalein sodium sulphonate (bromsulphalein) and tetrachloro(*P*)tetraiodo(*R*)fluorescein (Rose Bengal). Some of the halogenated phthaleins have unpleasant side-effects (Rosenau, 1925) and bromsulphalein is preferred in human work as it is non-toxic in ordinary doses and non-irritant. Supplies of this dye were unfortunately unobtainable at the time this work was carried out.

METHODS

Phenoltetrachlorophthalein Excretion Test. 0·1 ml. per kilo live weight of a 5 per cent solution of the dye (B.D.H. biologically tested) in normal saline was given into the jugular vein. Blood samples were taken from the opposite vein prior to and 2, 15 and 30 minutes and 1 and 2 hours following the injection. The concentration of the dye in the 2 minute sample was taken as the initial concentration, thus avoiding the necessity to assume, as in the original method (Rosenthal, 1922), that plasma volume is 50 ml. per kilo body weight. Extraction of the dye with acetone was necessary in some cases owing to slight lipaemia; extraction is not complete according to Bloom and Rosenau (1924).

Rose Bengal Test. The dye (B.D.H. adsorption indicator) was used in the form of a 2 per cent solution in normal saline, being given intravenously at the rate of 0·1 ml. per kilo live weight. The injection was made into the jugular vein and blood samples were withdrawn from the opposite vein immediately before and exactly 2, 8, 14 and 20 minutes after the injection. In the original technique, 6 ml. of acetone are added to 3 ml. of plasma or serum in order to precipitate the proteins and extract the dye. This procedure has been found not always necessary, but must be employed if the samples are haemolysed or turbid.

Since Rose Bengal is photosensitizing, cattle must be kept out of direct sunlight for some hours after carrying out the test.

RESULTS

Fig. 1 and Table I illustrate the results obtained with the phenoltetrachlorophthalein test in three normal cattle, in three animals showing severe diffuse hepatic fibrosis due to repeated doses of carbon tetrachloride (Garner, 1952) and in a bull possessing a fatty liver as a result of *Trypanosoma vivax* infection.

The rate of removal of Rose Bengal from the blood of seven normal cattle, two with mild and four with severe diffuse hepatic

TABLE I

THE PHENOLTETRACHLOROPHTHALEIN EXCRETION TEST IN CATTLE.

Animal No.	*Percentage of 2 min. concentration of dye remaining in serum after :* 15 min.	30 min.	1 hr.	2 hrs.
Normal animals.				
1126	11·1	9·6	nil	nil
1175	20·5	10·7	nil	nil
1191	14·2	10·0	nil	nil
Diffuse hepatic fibrosis (repeated doses of carbon tetrachloride).				
1146	11·6	8·5	nil	nil
1151	12·8	10·5	6·7	nil
1152	10·6	7·7	2·9	nil
Fatty changes (*Trypanosoma vivax*).				
1140	18·3	8·9	nil	nil

TABLE II

THE ROSE BENGAL TEST IN CATTLE.

Animal No.	*Normal Function* %
Normal animals.	
924	108
938	100
947	56
955	112
971	128
1126	116
1175	116
	(Mean, 108)
Diffuse hepatic fibrosis, Mild.	
952	98
974	108
	(Mean, 103)
Severe.	
1032	114
1146	82
1151	116
1152	72
	(Mean, 96)
Fatty liver (*Trypanosoma vivax*).	
1140	74

FIG. 1.

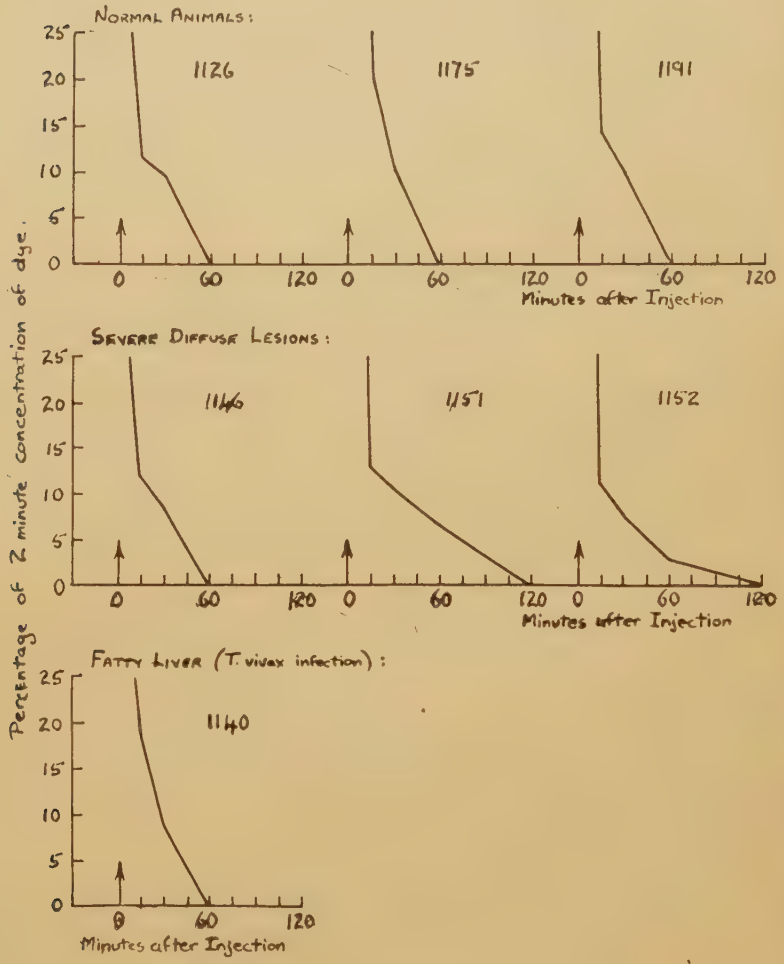

FIG. 1. The Phenoltetrachlorphthalein test in cattle.

fibrosis produced by carbon tetrachloride and one animal with trypanosomiasis is shown in Fig. 2. Table II lists the percentage normal liver function in these animals calculated on the basis that, if the dye concentration in the 8 minute sample is more than 50 per cent of that in the 2 minute sample, the liver is considered to be abnormal. Thus: —

$$\text{per cent normal function} = 200 - \frac{200\,R_2}{R_1}$$

where R_1 is the concentration in the 2 minute sample and R_2 that in the 8 minute sample.

FIG. 2.

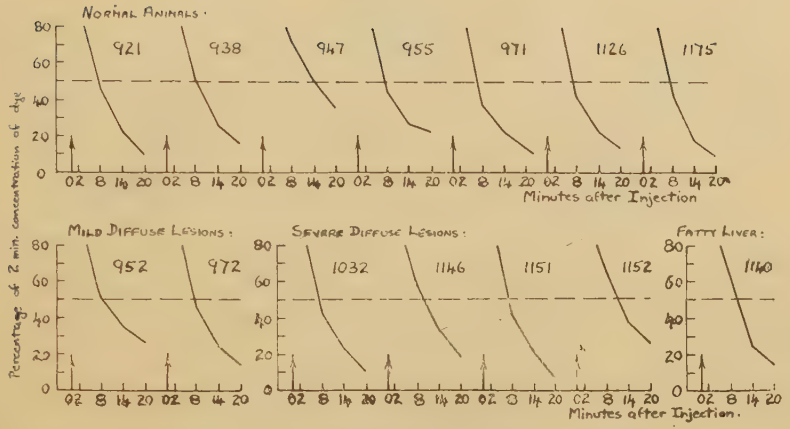

FIG 2. The Rose Bengal excretion test in cattle.

DISCUSSION

In man the concentration of phenoltetrachlorophthalein in the 1 hour sample is usually taken as the criterion of the existence of liver disease. Normally less than 3 per cent of the dye is left in circulation after this time, retention of 4 to 8 per cent indicates partial insufficiency, of over 8 per cent pronounced insufficiency (Rosenthal, 1922a; Bogen, 1923), while in severe liver disease as much as 50 per cent may still be in circulation (Harrison, 1947). In two animals (1151, 1152), some dye was still present after 1 hour. Both had been given 10 ml. of carbon tetrachloride on alternate days for 15 weeks and had massively fibrosed livers, nevertheless the rate of removal of the dye was not markedly different from that in the control animals. Moreover, No. 1146 had received the same treatment and yet showed no evidence of reduction in excretory capacity whatever.

The limited number of results reported here entirely support the conclusion of Hornby (1924) that the phenoltetrachlorophthalein test is of no value in cattle even with extreme impairment of liver function.

In the case of the Rose Bengal test, again, it is apparent that, even in the presence of severe hepatic fibrosis, normal function, as estimated by dye excretion, can be maintained (Nos. 1032, 1151). A larger series of cases might have indicated more precise differences in behaviour of normal and diseased cattle; on the evidence available the test must be considered unreliable.

It may be inferred from these results that the excretory capacity of the bovine liver, unlike that of the human liver, is relatively unaffected even by gross hepatic damage. This contention is supported by observations (Garner, unpublished) on bile pigment metabolism in liver disease.

SUMMARY

The rates of removal of phenoltetrachlorophthalein and Rose Bengal from the blood of cattle have been determined in cattle with and without liver damage.

It is concluded that dye excretion tests are unreliable as indicators of hepatic disease in cattle.

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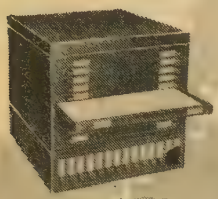
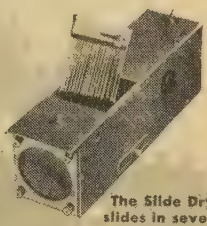
more than a mere laboratory instrument . . . . . .

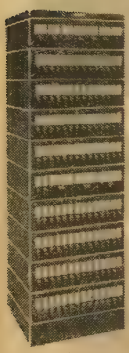


The Block File stores paraffin blocks with utmost economy of space

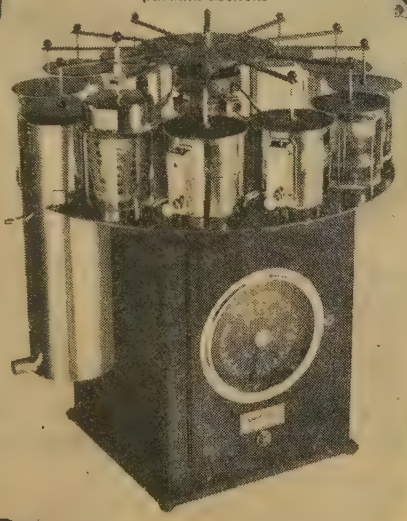
The constant-temperature Water Bath for floating paraffin sections

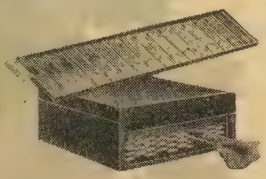


The Slide Dryer dries slides in seven minutes



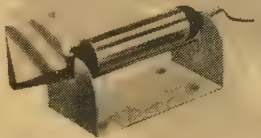
The Slide-files for all types of slides and transparencies



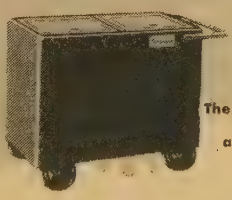


The Laboratory Slide Trays and Cabinet

The standardized reagents for tissue processing and staining



The electric Paraffin Knife for cutting, blocking and mounting tissues



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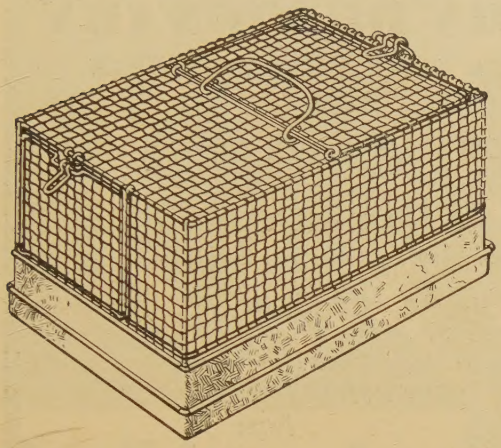

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